

***MOLECULAR STRATEGIES FOR THE DETECTION
OF MEASLES VIRUS IN
INFLAMMATORY BOWEL DISEASE***

**A thesis submitted for the degree of
Doctor of Philosophy**

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ABSTRACT

Hypotheses. i) Atypical exposure to measles virus is a factor in the aetiology of inflammatory bowel disease (IBD). ii) Measles, mumps and rubella (MMR) vaccination is a factor in the aetiology of autistic enteropathy.

Aims. i) To compare a range of molecular techniques for measles RNA amplification. ii) To develop a sensitive and robust method for the detection of measles RNA. iii) To analyse clinical samples from IBD patients for the presence of measles RNA. iv) To analyse clinical samples from autistic enteropathy patients for the presence of measles, mumps and rubella RNA.

Methods development. Three RNA amplification methods were compared in terms of their sensitivity and fidelity for the detection of measles RNA and nucleic acid sequence-based amplification (NASBA) was found to be the most sensitive. In a preliminary study, NASBA did not detect any measles RNA in a coded series of IBD and control intestinal tissues.

In order to improve the detection sensitivity, the use of hybrid capture, using measles-specific oligonucleotides linked to paramagnetic solid phase supports, was investigated. Hybrid capture was found to increase the measles RNA detection sensitivity 100-fold when followed by RT-PCR. An internal modified transcript was developed which could be co-amplified with measles RNA as an internal positive control.

IBD samples. Resection samples from 20 IBD and control patients were used for measles hybrid capture followed by RT-PCR, in addition to peripheral blood mononuclear cells (PBMCs) from 13 IBD and control patients.

Autistic enteropathy samples. Biopsies, PBMCs and Vero/PBMC cocultures were analysed from 22 patients with autistic enteropathy and 6 controls.

Results. Hybrid capture and RT-PCR could detect 10^4 molecules of a measles RNA transcript added to control tissue homogenates. The fidelity of NASBA, in terms of its nucleic acid error rate, was found to be comparable with that of RT-PCR. All samples were found to be positive for a housekeeping RNA species and internal modified positive control RNA. None of the samples tested positive for measles, mumps or rubella RNA, although viral RNA was successfully amplified in positive control samples.

Conclusion. The results do not support previous data implicating persistent measles virus infection with the aetiology of IBD or autistic enteropathy.

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LIST OF ABBREVIATIONS

AMV	Avian myoblastosis virus
ANCA	Anti-neutrophil cytoplasmic antibody
AIH	Autoimmune hepatitis
bp	base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	complementary DNA
ddNTP	dideoxynucleotide triphosphate
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DNase	Deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethalynediamine tetra-acetic acid
ELGA	Enzyme-linked gel assay
FBS	Fetal bovine serum
H-gene	Haemagglutinin gene
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IBD	Inflammatory bowel disease
IL	Interleukin
IFN	Interferon
kb	kilobase
LB	Luria-Bertani
MIBE	Measles inclusion body encephalitis
MMLV	Moloney murine lymphoma virus
MMR	Measles, mumps and rubella
MOPS	Morpholino-propanesulphonic acid
mRNA	messenger RNA
MSPS	Magnetic solid phase support

N-gene	Nucleoprotein gene
NASBA	Nucleic acid sequence-based amplification
nt	nucleotide
NTP	Nucleotide triphosphate
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
PVP	Polyvinylpyrrolidone
RNA	Ribose nucleic acid
RNase	Ribonuclease
RT	Reverse transcription
SDS	Sodium dodecyle sulphate
SSC	Saline sodium citrate
SSPE	Subacute sclerosing panencephalitis
TEMED	Triethylethylenediamine
Th	Helper T cell
TNF	Tumour necrosis factor
Tris	Tris-hydroxymethyl-aminomethane

GENERAL INTRODUCTION

Crohn's disease and ulcerative colitis are chronic inflammatory bowel diseases (IBD) of unknown aetiology. It is widely believed that IBD results from either a normal immune response to a particular infectious agent, or an abnormal immune response to enteric flora.

One hypothesis is that a persistent measles virus infection, originating from early or atypical exposure to measles virus, may be involved with the development of IBD - particularly Crohn's disease. Several epidemiological studies by our group and others have shown early measles infection and measles vaccination to be risk factors for the development of IBD. Furthermore, several electron microscopy and immunohistochemical studies by our group and others have provided evidence of measles virus in areas of granulomatous inflammation within IBD tissue. However, such evidence remains controversial and ongoing research by our group aims at resolving this matter.

In order to confirm results from electron microscopy and immunohistochemical studies, it is essential to detect measles virus RNA in IBD tissue using highly sensitive molecular biology techniques, such as nucleic acid amplification.

Given that previous studies have failed to detect measles RNA in IBD clinical samples, the aims of the studies described in this thesis were to develop a highly sensitive method capable of detecting low copy-number measles RNA in clinical samples. This could then be applied to the detection of measles RNA in clinical samples from IBD patients in order to provide evidence for or against the involvement of measles virus in the disease aetiology.

Research at the Royal Free Hospital has focused on early exposure to measles virus as a factor in the aetiology of IBD, particularly Crohn's disease and therefore, the molecular studies described in this thesis concentrate primarily on measles virus and Crohn's disease.

In addition, a group of paediatric patients, presenting with ileo-colonic lymphoid hyperplasia, chronic colitis and developmental disorder (autistic enteropathy) - temporally associated with measles, mumps and rubella (MMR) vaccination - *has* have been recently described in a study at the Royal Free Hospital. These patients may represent cases of early-onset IBD and therefore experiments

described in this thesis, concerning the detection of measles RNA (in addition to mumps and rubella RNA) in clinical samples from these patients, may provide further evidence for or against the involvement of measles virus in the disease aetiology.

The first chapter of this thesis gives a background to IBD, discussing the evidence for an infectious aetiology and the role of granulomatous vasculitis in the pathogenesis of Crohn's disease. The second chapter summarises the evidence for and against the involvement of measles virus in the aetiology of IBD and autistic enteropathy. The third chapter describes measles virus, diseases associated with measles virus, and methods used to detect measles virus RNA.

Following the materials and methods chapter (Chapter 4) and the results chapters (Chapters 5-10), the implications of the findings of this study, with regard to measles virus, IBD, and autistic enteropathy, are discussed in detail (Chapter 11).

Chapter 1

Chapter 1: INFLAMMATORY BOWEL DISEASE

Evidence that ulcerative colitis was first recognised as a disease entity in the mid 19th century exists in several case reports, although a definitive clinical description of the disease was not given until 1921 in a Guy's Hospital report by Sir Arthur Hurst.

Inflammation of the terminal ileum was described by the Scottish surgeon, Kennedy Dalziel in 1913, but it was Burrill Crohn *et al.* who presented a report on the clinical and pathological features of regional ileitis to the American Medical Association in 1932 (Crohn *et al.* 1932). Although Ginsberg and Oppenheimer contributed equally to the work, Crohn appeared as first author on the paper published by the Journal of the American Medical Association by virtue of the journals' alphabetical listing of authors and thus "terminal ileitis" or "regional ileitis" subsequently became known as "Crohn's disease".

IBD usually occurs in early adulthood and is characterised clinically by abdominal pain, weight loss and diarrhoea (containing blood and mucus), and often requires long-term anti-inflammatory therapy (steroid and/or non-steroid drugs; reviewed by Jewell, 1990; Rhodes and Mayberry, 1990). Wide variations occur between patients with IBD in terms of the site and clinical course of disease. It is thought by some that ulcerative colitis and Crohn's disease may represent different ends of an IBD spectrum (with cases of indeterminate colitis possessing features of both diseases), although there are important differences between classical Crohn's disease and ulcerative colitis in terms of their epidemiology, pathology and clinical course.

Periods of remission and relapse are a feature of both diseases, and surgery is often required to remove sections of affected gut if the disease becomes unresponsive to steroid or other immunosuppressive therapy. Although surgical intervention may prevent further disease in patients with ulcerative colitis, it often does not prevent future disease relapse in patients with Crohn's disease. Moreover, surgical intervention often leads to absorption and nutritional problems associated with short bowel syndrome.

In recent years the incidence of IBD has increased dramatically, particularly in childhood, without many significant improvements in disease treatment, posing

a major health problem to western societies.

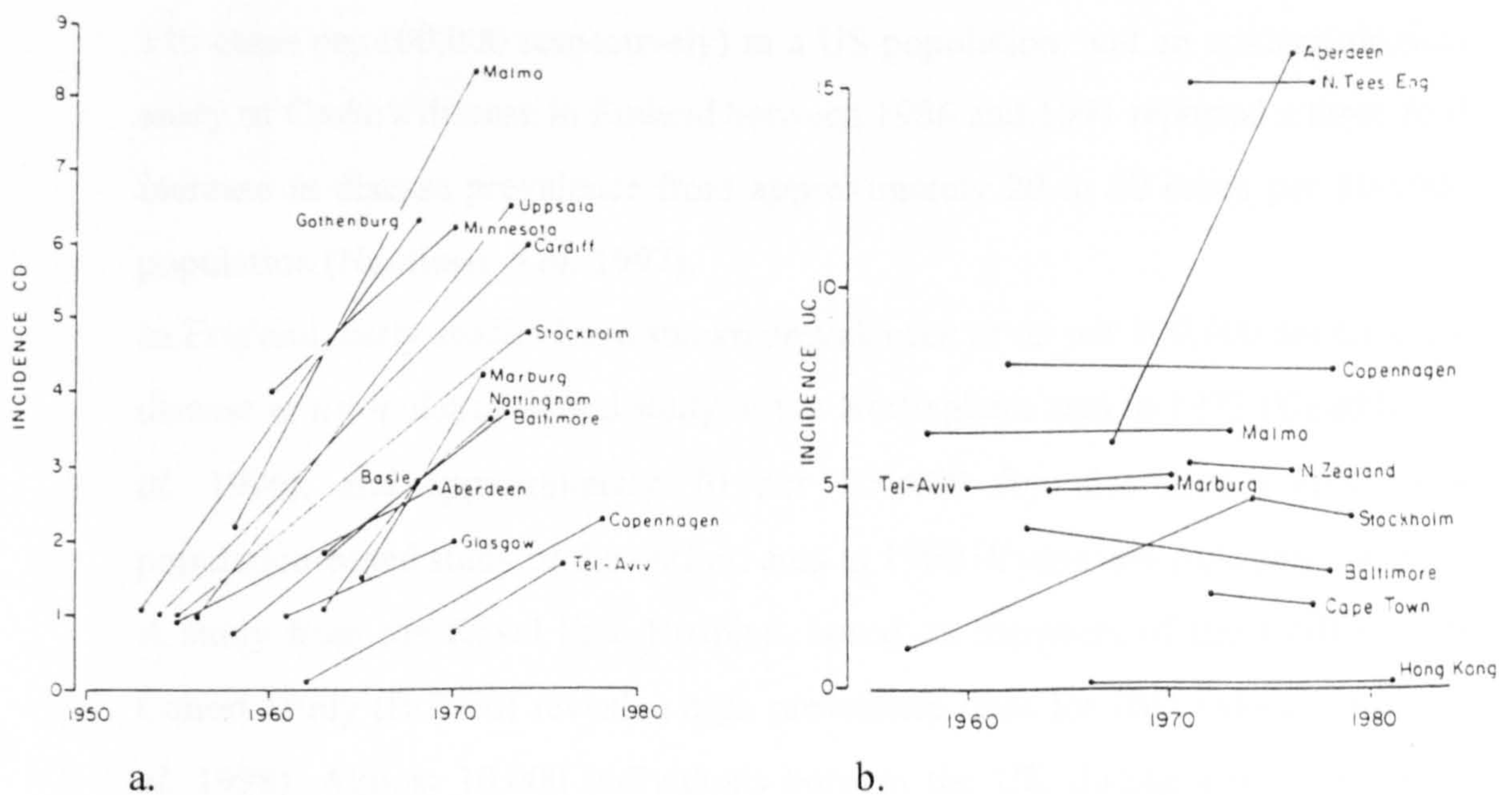
Regarding the aetiology of IBD, much early work focused on a psychosomatic origin of the disease, and the possibility that IBD was a stress-related disorder was popular for a long time. More recently, several aetiological factors have been proposed, ranging from genetic factors responsible for gut immune regulation, to infectious agents such as *Mycobacteria paratuberculosis* and measles virus. However, decades after the first descriptions of IBD, the relative merits of many proposed aetiological factors remain widely debated and the huge volume of research into IBD has not led to a foreseeable discovery of a cause or cure.

1.1. THE EPIDEMIOLOGY OF INFLAMMATORY BOWEL DISEASE

1.1.1. Prevalence and Incidence

Incidence. Several studies have shown that the incidence of IBD is increasing in developed countries (reviewed by Langman (1990) and Sandler (1994)). In general, early reports showed a higher incidence of ulcerative colitis compared to Crohn's disease whereas more recent reports have shown a higher incidence of Crohn's disease.

Figure 1.1: The increasing incidence of Crohn's disease.



Rise in incidence of Crohn's disease (a) compared to the relatively steady incidence of ulcerative colitis (b) reported in several studies. The y axis represents incidence per 100,000 population. Data was taken from a review by Langman (1990) with permission from Churchill Livinstone Press.

It is possible that these trends may, in part, be artifactual. However, the large number of studies performed on different populations support the phenomenon of increasing incidence and prevalence of IBD, and it is therefore unlikely that artifactual data alone are responsible for this trend.

Mayberry *et al.* (1979) reported a dramatic increase in the incidence of Crohn's

disease in a population in Cardiff, UK, from 0.18 to 4.8 cases per 100,000 per annum between 1934 and 1977 (see Figure 1.1.a.). Of particular interest in this study, the authors demonstrated that neither disease mis-diagnosis, nor changes in diagnostic practice, alone were likely to be responsible for this rise in incidence by following up reported cases and showing that disease reclassification did not alter the original findings.

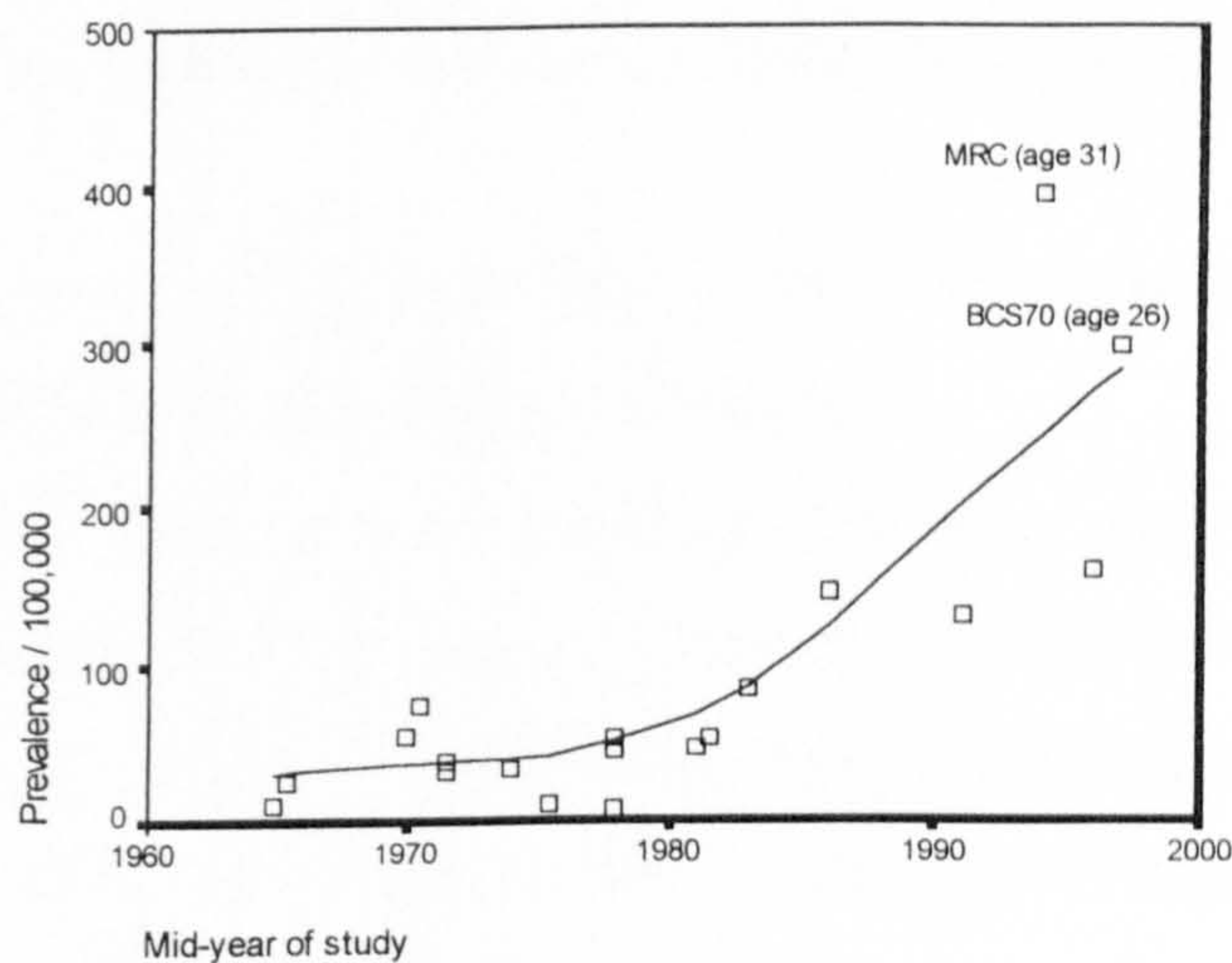
Prevalence. In general, recent studies have shown much higher prevalence rates for IBD than early studies (Sandler 1994). For instance, Loftus *et al.* (1997), observed high prevalence rates of Crohn's disease and ulcerative colitis (104 and 116 cases per 100,000 respectively) in a US population, and an epidemiological study of Crohn's disease in Finland between 1986 and 1991 reported a three-fold increase in disease prevalence from approximately 20 to 60 cases per 100,000 population (Nuutinen *et al.* 1997).

In England, early studies have shown prevalences of 65 per 100,000 for Crohn's disease in a population-based study of the Nottingham area in 1973 (Keighley *et al.* 1976), and approximately 70 per 100,000 for ulcerative colitis in a population-based study of the Oxford area in 1960 (Evans and Acheson, 1965).

A study from the Royal Free Hospital, based on members of the 1970 British Cohort Study (BCS70) revealed high prevalence rates for IBD (Montgomery *et al.* 1998). Almost 10,000 individuals born in the UK during a week in 1970 replied to a questionnaire concerning IBD diagnosis. The overall prevalence of IBD in this population was found to be 520 per 100,000, with the prevalences of 306 and 214 per 100,000 for Crohn's disease and ulcerative colitis, respectively. These prevalence rates represent a dramatic increase compared to those previously found in English populations, and since the members of the BCS70 cohort were only 26 years of age at the time of study, it is likely that prevalence rate will further increase as the cohort ages. The increasing prevalence of Crohn's disease is represented in Figure 1.2.

The dramatic increase in the incidence of IBD, particularly Crohn's disease, argues against a strong genetic component in the aetiology of this disease since such a genetic factor could not become so widespread in a period of decades.

Figure 1.2: The increasing prevalence of Crohn's disease.



The increasing prevalence of Crohn's disease over time with boxes representing the results of published prevalence studies. MRC represents the prevalence rate in the study by Thompson *et al.* (1995a) and BCS70 represents the prevalence rate in the study by Montgomery *et al.* (1998). Data supplied by Dr. A Wakefield (personal communication).

IBD in Childhood. The incidence of childhood IBD, in particular Crohn's, has increased since the mid 1970's (Barton *et al.* 1993; Cosgrove *et al.* 1996), again indicating the importance of environmental risk factors. Candidate environmental factors include the measles vaccination program which began in 1969 and evidence for this will be discussed later.

In 1996 Cosgrove *et al.* reported a 2.5-fold increase in the incidence of Crohn's disease in the UK during an 11 year period, contrasting with a constant incidence rate for ulcerative colitis. The corresponding IBD prevalence rate at the end of the study period was 16.6 cases per 100,000 with Crohn's disease accounting for 83% of these cases, emphasising the emergence of Crohn's disease in childhood .

1.1.2. Age

The peak age of onset of IBD has been shown to be between approximately 15 and 25 years (Sandler *et al.* 1994) although the disease can develop at any age. This delayed onset of disease is unlikely to be solely due to genetic factors and three possible mechanisms for this delay are outlined below:

- i) A genetic susceptibility to IBD may require an environmental trigger later in life for the development of disease.
- ii) Exposure to an environmental agent early in life may require a second exposure for disease development. For instance a persistent microbial infection early in life may require an acute or sub-clinical infection with the same or similar agent later in life to induce a chronic inflammatory response to the persistent infection.
- iii) Exposure to an environmental agent early in life may induce a dysregulation of the host immune system which only manifests itself following an infection of, or physical damage to, the intestine later in life.

Several studies have demonstrated a bimodal age distribution of IBD incidence, with a second peak in elderly age groups and it has been suggested that this second peak could be due to a persistent environmental agent which is activated in later life (Sandler, 1994). Alternatively, increased susceptibility to vascular disease with age may account for this second peak if chronic granulomatous vasculitis plays a role in the early pathogenesis of Crohn's disease, as has been suggested (Wakefield *et al.* 1991a).

1.1.3. Sex and Social Class

Several studies have shown that females may be more at risk of developing Crohn's disease, and males more at risk of developing ulcerative colitis and an increased incidence of IBD in higher socio-economic groups has also been reported (reviewed by Langman, 1990). However, a more recent study from our group found no association between sex or socio-economic class and the prevalence of IBD (Montgomery *et al.* 1998), indicating that these factors have become less important risks for development of IBD over the past five decades,

and that the incidence of IBD is increasing throughout society. Therefore, any genetic components involved with the development of IBD via sex and social class have become overshadowed by the dramatic increase in the incidence of IBD in both sexes and all social classes.

One explanation for this is that there may previously have been differences between sexes and social classes, in terms of susceptibility to an infectious agent involved with the aetiology of IBD. As living standards improved, it may be assumed that exposure to such an agent was reduced leading to a reduction IBD incidence. However, if vaccination for such an agent is also involved with disease aetiology, then universal vaccination may lead to an increase in IBD incidence in all areas of society as the epidemiological data suggests.

1.1.4. Distribution

IBD is predominantly a disease of developed societies and has generally been reported to be more common in urban versus rural areas (Kyle, 1971a; Ekblom *et al.* 1991a). This may be due to a lower level of domestic hygiene in urban areas due to a greater population density, leading in urban areas to an altered pattern of exposure of an infectious agents. For instance, an infectious agent which may be involved with the aetiology of IBD may spread rapidly through an urban community leading to the infection of many individuals early in life - a factor which may be important in IBD aetiology. In rural communities however, such an infection may not spread as rapidly and therefore individuals may become infected later in life.

This mechanism has been supported by Gent *et al.* (1994) who described an association between childhood domestic hygiene and the development of IBD.

1.1.5. Perinatal risk factors

Several studies have shown events early in childhood to be important for the later development of IBD. Ekblom *et al.* (1990), studying a Swedish population, showed that patients with IBD, or their mothers, were more likely to have had a recorded health event during or after their pregnancy compared to controls (odds ratio = 4.4). Another study by the same research group (Ekblom *et al.* 1991b) found evidence for clustering of IBD in terms of birth place and time, and

seasonal peaks in the incidence of ulcerative colitis (but not Crohn's disease). These results imply that exposure to an environmental agent(s) during or after pregnancy may be involved with the development of IBD. Other studies have confirmed that perinatal factors are important in the development of IBD (Gent *et al.* 1994; Thompson *et al.* 1995a; Montgomery *et al.* 1997), further arguing against a strong genetic component in this disease.

1.1.6. Childhood Factors

Domestic Hygiene. In a 1994 UK study, Gent *et al.* (1994) demonstrated a correlation between childhood domestic hygiene and the later development of Crohn's disease. Children whose first house contained a hot water tap (used in this study as a marker for domestic hygiene) were found to be at a greater risk of developing Crohn's disease than those whose first house did not. The authors argued that improved domestic hygiene could lead to a decreased exposure to certain infectious agents early in life. The authors also suggested that delayed exposure to enteric infections could trigger an abnormal immune response, leading to chronic inflammation. This study found no association between childhood domestic hygiene and the development of ulcerative colitis, indicating that different aetiologies may be responsible for the two diseases, or that different patterns of exposure to an infectious agent may lead to the development of different IBD phenotypes.

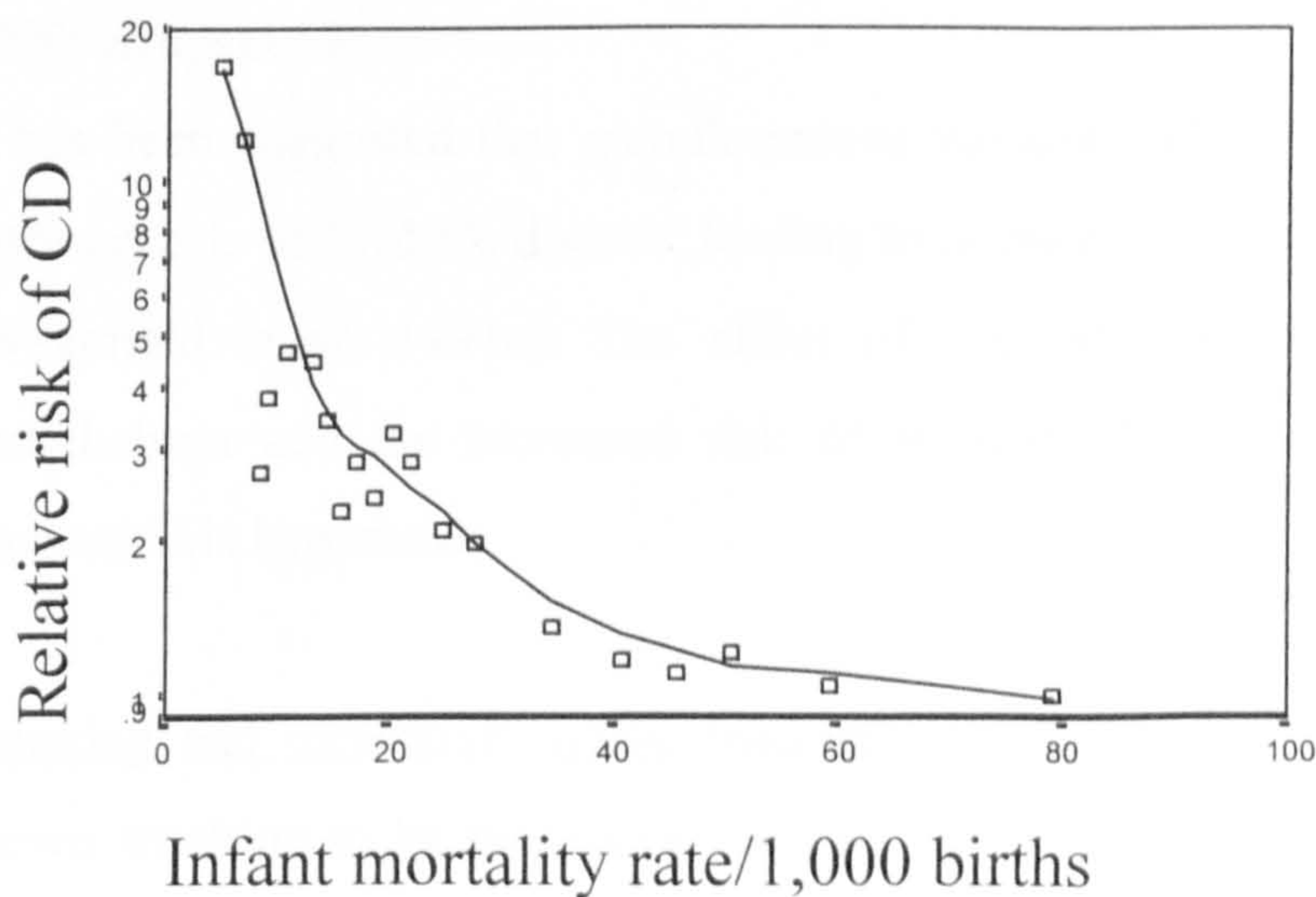
There are interesting parallels with regard to age of exposure to infectious agents and the development of IBD. Delayed exposure to Epstein-Barr virus (EBV) has been shown to be a risk factor for the development of Hodgkin's disease, a malignant lymphoma. In 1981, Gutensohn and Cole identified a number of risk factors for the development of Hodgkin's disease. It was shown that risk increased with age of exposure to infectious agents since patients with the disease generally had less contact with children of the same age group than controls. From this data, the authors suggested that delayed exposure to EBV may be responsible for the associated cases of Hodgkin's disease.

Another example of delayed exposure to a virus leading to complications later in life is that of polio virus. Poliomyelitis, a complication following acute infection occurs in approximately 1% of infected individuals and it has been shown that

the severity of this complication increases with age of exposure with severe paralysis occurring in some individuals (Gutensohn and Cole, 1977). Interestingly, several studies have reported the occurrence of poliomyelitis following vaccination (Smith and Wherry, 1978; Strebel *et al.* 1994), providing a possible parallel for measles vaccination and the development of Crohn's disease. There are striking similarities between Gutersohn and Cole's studies on Hodgkin's disease and poliomyelitis, and age of virus exposure, and that of Gent *et al.* which supports the hypothesis that delayed exposure to an infectious agent may be involved with the aetiology of Crohn's disease.

Infant mortality. In study at the Royal Free Hospital, Montgomery *et al.* (1997) showed an inverse relationship between infant mortality and incidence of IBD throughout Europe, with Portugal having the highest rate of infant mortality and the lowest incidence of IBD, and countries such as The Netherlands having the lowest rate of infant mortality and the highest incidence of IBD. These results have been supported by a study of the incidence of IBD in Iceland from 1915 to 1984, showing a relative risk of 17 for the development of Crohn's disease at the time of lowest infant mortality compared with the time for highest infant mortality (Montgomery, S.M., personal communication).

Figure 1.3: Infant mortality and Crohn's disease.



The relative risk of Crohn's disease compared to infant mortality rates in Iceland. Data supplied by Dr S. Montgomery (personal communication).

However, this relationship was not as strong in ulcerative colitis. The authors argued that in the case of Crohn's disease, an environmental aetiological agent may be present which is fatal during times of high infant mortality (Montgomery *et al.* 1997). Therefore, as living standards improve, more infants survive early infection to develop persistent infection associated with Crohn's disease. This may explain the findings of Gent *et al.* in as much as there is an inverse relationship between levels of childhood domestic hygiene and infant mortality. Improvements in living conditions may have altered the pattern of exposure to such an infectious agent leading to an increased frequency of persistence, and the development of IBD. Alternatively, an environmental aetiological agent which has become more prominent as living conditions have improved could also account for this phenomenon.

1.1.7. Adult factors

Smoking and Crohn's disease. Studies have shown relative risks for the development of Crohn's disease among current smokers to range between 1.2 and 3.9, and several studies have found smoking to have a potentiating effect on Crohn's disease (reviewed by Sandler 1994), suggesting that smoking is involved with both the development and potentiation of Crohn's disease.

Smoking has been shown to induce endothelial cell injury, leading to the formation of microthrombi, and enhanced platelet aggregation (Pittilo *et al.* 1990; Krupski 1991).

It has been suggested that granulomatous vasculitis plays an early role in the pathogenesis of Crohn's disease, leading to ischaemia and chronic inflammation (Wakefield *et al.* 1991a). The effect of cigarette smoking on the vascular endothelium and the increased risk of smokers developing Crohn's disease supports this hypothesis.

Smoking and ulcerative colitis. Interestingly, data from several studies have shown smoking to be protective for the development of ulcerative colitis. For example, Lindberg *et al.* (1988) reported a relative risk of 0.7 for the development of ulcerative colitis among smokers compared to non-smokers, and a relative risk of 12.2 for the development of ulcerative colitis in former smokers

compared to smokers has been shown (Thornton *et al.* 1985), confirming the protective effect of smoking in ulcerative colitis. In addition, several studies have shown that smoking protects against disease relapse in ulcerative colitis in a dose-dependent manner (Franceschi *et al.* 1987; Lindberg *et al.* 1988) indicating a protective role in disease pathogenesis.

Colonic mucus composition and production in patients with ulcerative colitis has been shown to be abnormal (Podolsky and Isselbacher, 1983; Cope *et al.* 1986) and may be involved with diseased pathogenesis. As proposed by Rhodes (1989), abnormal colonic mucus secretion could expose the colonic epithelium to invasive bacteria, eliciting an inflammatory immune response. Nicotine has been shown to increase colonic mucus production (Finnie *et al.* 1996) and it has been suggested that the effects of cigarette smoking may compensate for abnormal mucus secretion in individuals susceptible to ulcerative colitis leading to disease remission in these patients (Thomas *et al.* 1996). Therefore, removal of this compensatory mucus secretion may then lead to the development of ulcerative colitis or disease relapse.

Such studies on smoking and IBD indicate that different aetiological and pathological mechanisms may be involved with the development of these diseases. Alternatively, it is possible that a chemical in tobacco such as nicotine may modulate the immune response in different ways, leading to an up-regulation of the host inflammatory response in Crohn's disease and a down-regulation in ulcerative colitis.

Oral contraceptives and IBD. Women taking oestrogen-containing oral contraceptives have a higher risk of developing IBD (Boyko *et al.* 1994). Oral contraceptives have been shown to have prothrombotic properties (Beller and Ebert, 1985) and it has been proposed that these thrombotic effects of the pill lead to the occlusion of intestinal submucosal blood vessels in susceptible individuals which, in turn leads to the pathological events of Crohn's disease (Wakefield *et al.* 1991b).

1.1.8. Conclusions

Recent studies have shown a dramatic increase over time in the prevalence of

IBD, particularly Crohn's disease, compared to previous studies, indicating that environmental factors are important in disease development. A number of childhood factors, among them childhood domestic hygiene and infant mortality, have been shown to influence the incidence of IBD. In addition, studies by Ekblom and his co-workers have indicated that childhood infections are important in the development of IBD.

Significant differences between Crohn's disease and ulcerative colitis, in terms of risk factors, suggest that either different aetiologies are responsible for the development these diseases, or that a changing pattern of exposure to a common aetiological agent determines the phenotype of disease.

Taken together, epidemiological studies argue against a strong genetic component in the aetiology of IBD and suggest that infectious agents, and particularly altered exposure to such agents may be important in disease development.

1.2. THE IMMUNOGENETICS OF INFLAMMATORY BOWEL DISEASE

Epidemiological studies have shown a higher incidence of IBD among Caucasians, especially Jewish populations, than other racial groups and a high incidence of IBD among Jews in different geographical areas has been shown (Reviewed by Yang and Rotter 1994). In the light of such evidence, there is little doubt that genetic factors are involved with susceptibility to IBD. However, the high prevalence of IBD in Jewish populations may be confounded by factors such as socio-economic status and infant mortality, and the size of the genetic contribution is debatable. The mode of inheritance of such a genetic factor is unlikely to be simply Mendelian, and the observed heterogeneity of IBD in terms of clinical course and severity indicates that a complex combination of genetic elements in addition to environmental factors are involved in both disease susceptibility and clinical course.

1.2.1. Familial studies

Several studies have shown an increased risk of the development of IBD in the relatives of individuals with IBD (Mayberry *et al.* 1980; Fielding, 1986). Such increased risks may represent a common exposure to an environmental agent or transmission of agents from mother to child, rather than a strong genetic component to the disease.

Several studies have reported families with multiple individuals affected with IBD (Kirsner, 1973; van Kruiningen *et al.* 1993). van Kruiningen *et al.* (1993) reported two French families with multiple affected individuals with Crohn's disease. Development of disease appeared to have occurred in 7-13 month time periods and the authors concluded that an infectious micro-organism, rather than inheritance of susceptibility genes, was the most likely cause for these particular cases.

No increase in the incidence of IBD has been observed in spouses of individuals with IBD compared to the general population (Mayberry *et al.* 1980; Weterman and Pena, 1984), arguing against a rapid infectious aetiology for IBD, and suggesting the involvement of genetic factors and/or early environmental factors

in disease development.

1.2.2. Twin studies

Twin studies represent a valuable source of data for determining the contribution of genetic elements to the development of IBD. Differences between monozygotic and dizygotic twins pairs, in terms of whether either one or both twin is affected, enables the extent of the genetic contribution of IBD to be determined, since twins have presumably been exposed to very similar environmental factors. Differences in disease concordance between affected twin pairs enables the differences in inheritability between Crohn's disease and ulcerative colitis to be analysed.

Tysk *et al.* (1988), in a study of 80 affected twin pairs in the Swedish twin registry reported a higher rate of disease concordance among monozygotic twins (one of 16 twins for ulcerative colitis, and 8 of 18 twins for Crohn's disease) than dizygotic twins, indicating that genetic factors are important in the development of IBD, particularly in Crohn's disease and possibly accounting for the observed familial aggregation of IBD. However, in a similar study at the Royal Free Hospital, Thompson *et al.* (1996) reported IBD concordance in 11 of 63 identical twin pairs and 4 of 81 non-identical twin pairs. Although significant, the small difference in disease concordance between identical and non-identical twins in this study indicates that environmental factors are more important than genetic factors for the development of IBD.

Results from such twin studies have shown the coefficient of inheritability of Crohn's disease to be in the same range as the inheritability of diseases which are thought to be partly genetically determined such as diabetes and schizophrenia.

1.2.3. Genetic markers

Linkage analysis. Linkage analysis studies have shown several genetic loci to be involved with the inheritance of IBD and its clinical course (Jewell, 1997). Many of these studies have focused on genes involved with the regulation of the immune system (such as human leukocyte antigen (HLA) complex genes) on the assumption that immune dysregulation is involved with both the susceptibility

and phenotypic expression of IBD.

However, the weakness of the association between these genes and disease susceptibility suggest that other non-HLA genes are also involved with disease susceptibility. It is possible that combinations of several genetic loci, involved with factors such as gut barrier function, immune regulation and repair of intestinal tissue following inflammation, may be involved with the development of IBD. In this manner, different combinations of many genetic susceptibility loci may lead to the development of a number of phenotypically similar inflammatory bowel diseases in certain individuals. Such a mechanism has recently been reviewed by Jewell (1997). These diseases are then clinically classified as ulcerative colitis, indeterminate colitis or Crohn's disease. The classification and characterisation of subsets of inflammatory bowel diseases (e.g. colonic Crohn's disease) may aid the discovery of genes conferring a significant susceptibility to the development of IBD.

Antineutrophil cytoplasmic antibodies (ANCA)s. ANCA)s have been demonstrated to be involved with diseases associated with vasculitis, for instance in Wegener's granulomatosis. Saxon *et al.* (1990) demonstrated the presence of ANCA)s in the sera of 84% of patients with ulcerative colitis and a subsequent study (Hertervig *et al.* 1995) demonstrated a higher prevalence of perinuclear ANCA (pANCA) in the sera of patients with colonic Crohn's disease (36%) than in those without colonic disease (18%). The target of these antibodies has not yet been determined and their significance in ulcerative colitis and colonic Crohn's disease remains unclear although the association with vascular disease is intriguing given the involvement of vasculitis in Crohn's disease (Wakefield *et al.* 1991a).

1.2.4. Bacteria

Recent work has shown that monocytes in the lamina propria of patients with IBD, but not in the lamina propria of control patients, express the bacterial lipopolysaccharide receptor CD14 (Grimm *et al.* 1995). The responsiveness of these cells to luminal bacterial antigens may be involved with the perpetuation of chronic intestinal inflammation.

It has been observed that in animal models susceptible to colitis (e.g. the interleukin (IL)-10 knockout mice), growth in germ-free conditions does not lead to the development of chronic colitis (Kuhn *et al.* 1993), indicating that bacterial luminal antigens are involved with the propagation of a chronic inflammatory response in these models. Jung *et al.* (1995) have shown that epithelial cells produce a range of proinflammatory cytokines in response to bacterial invasion *in vitro*, leading to the hypothesis that increased permeability of the gut may lead to invasion of epithelial cells by luminal bacteria and thus production of pro-inflammatory cytokines, giving rise to chronic inflammation. Such an increase in gut permeability may be genetically determined or may arise from previous damage caused by an infectious agent.

Hermiston and Gordon (1995) elegantly demonstrated the importance of gut barrier function in IBD using a dominant negative N-cadherin mouse model. N-cadherin is involved with cell adhesion and areas of the gut which did not express N-cadherin were found to be susceptible to inflammation due to the influx of luminal bacteria.

1.2.5. Immune dysfunction

IBD is an immune-mediated condition. Dysfunction of the gut immune system is thought to play an important role in IBD, both in terms of disease susceptibility and propagation of chronic inflammation and evidence for this has come from animal studies. Several rodent models - either transgenic or knockout for particular cytokine genes - develop colitis (reviewed by Elson *et al.*, 1995). In addition to cytokines themselves, mutations in, or altered expression of, cytokine receptors and proteins involved with cytokine intracellular signalling pathways may also lead to chronic inflammation.

A host of factors, including cytokines, chemokines, T and B cell costimulatory molecules, and molecules involved with T cell activation-induced apoptosis are involved with immune regulation and disruption of any one, or combinations of these factors may be important in the development of chronic inflammation.

Crohn's disease is immunologically characterised by cell mediated response to an unidentified antigen or set of antigens. During active inflammation, T-cells are recruited from peripheral blood via the production of endothelial cell

adhesion molecules. The different range of cytokines produced by peripheral blood T-cells compared to lamina propria T-cells may play a role in the propagation of chronic inflammation (Targan *et al.* 1995).

It has been shown that stimulation of peripheral T cells, but not lamina propria T-cells induces the production of the cytokines IL-2, IL-4, interferon gamma (IFN- γ) and tumour necrosis factor alpha (TNF- α) (Targan *et al.* 1995). Such cytokines are thought to play an important role in disturbing the immunological homeostasis of the gut in IBD.

In view of these findings, it is reasonable to assume that intestinal microvascular endothelial cells play a key role in the generation and propagation of chronic inflammation in IBD.

Th1/Th2 cytokine profiles. Disruption to the homeostasis of the gut immune system is thought to play a major role in both the aetiology and pathogenesis of IBD. Studies on cytokine profiles in IBD are complicated by the fact that most have looked at end-stage disease tissue samples (e.g. Niessner and Volk, 1995) which may contain abnormal levels of cytokines produced as a consequence of chronic inflammation rather than being involved with the development of colitis. The normal intestine is in a state of chronic low-level inflammation due to the array of bacterial, food and viral antigens constantly encountered in the gut lumen and the host response to these antigens is thought to involve a balance between Th1 and Th2 cytokines (Mosmann and Coffman, 1989).

Th1 cytokines (including IL-2, TNF- α and IFN- γ) are predominantly pro-inflammatory, inducing a cell-mediated response, i.e. macrophage activation and cell-mediated cytotoxicity, while Th2 cytokines (including IL-4, IL-5, IL-6 and IL-10) are generally protective, leading to macrophage deactivation and stimulation of a humoral response (Neurath and Meyer zum Buschenfelde, 1996).

Powrie *et al.* (1994) have studied the relationship between Th1 cells, Th2 cells and chronic colitis by reconstituting severe combined immunodeficient (SCID) mice with Th1 and Th2 cell populations (Powrie *et al.* 1994). This work showed that, in mice, Th1 cells are responsible for the development of a colitis which can be prevented by the presence of Th2 cells. Subsequent work identified

transforming growth factor β as the cytokine secreted by Th2 cells suppressing the Th1 cell-mediated inflammatory response in this model (Powrie *et al.* 1996). Such work on animal models of colitis has highlighted the importance of Th1 and Th2 responses in the development of chronic colitis.

IL-12, a cytokine produced by dendritic cells, is thought to mediate differentiation of Th0 cells to Th1 cells (Palmer and van Seventer, 1997) and may therefore be involved with the establishment of chronic inflammation. Bacterial infection of dendritic cells has been shown to induce IL-12 (Heufler *et al.* 1996) production and this may lead to the propagation of chronic inflammation following bacterial invasion of lymphoid tissue.

1.2.6. Oligoclonal T cell response

In an elegant approach to determine the nature of antigens responsible of the inflammatory response in IBD, Chott *et al.* (1996) analysed the T cell receptor (TCR) gene region of lamina propria CD4+ and CD8+ cells, using PCR. Lamina propria T cells from control tissue showed a wide range of amplification products indicating that no amplification of a specific T cell clone had occurred. However, cells from ulcerative colitis patients produced a smaller range of PCR products, indicating the expansion of certain T cell clones. The TCR binding site peptide sequences of these expanded T cell clones were found to be similar both within and among patients, indicating an oligoclonal response to a common antigen. Similar results using T cells isolated from patients with Crohn's disease are currently underway. These experiments raise the possibility that a single antigen, or a group of closely related antigens, are the focus of a chronic inflammatory response in patients with IBD.

1.2.7. Conclusions

Genetic studies have shown that polygenic inheritance is involved in IBD susceptibility and clinical course. Such genes have not as yet been characterised but are likely to be involved with regulation of the intestinal immune system and gut barrier function. However the weak associations reported, together with the data from twin studies, indicate that environmental factors are more important in determining IBD susceptibility.

Immunological evidence points to an immune dysregulation in IBD in conjunction with a oligoclonal T cell response, possibly to a single antigen, leading to the propagation of chronic intestinal inflammation. The imbalance between Th1- and Th2-type T cells may be due to infection of cells involved with Th0 cell differentiation, for instance dendritic cells, and may disrupt intestinal homeostasis, leading to chronic intestinal inflammation.

It is likely that an infectious agent (or group of agents) is involved with the initial damage to the gut barrier required to generate an inflammatory response. Susceptibility to infection, failure to repair damaged intestinal tissue, and failure to downregulate the inflammatory response in some individuals may lead to the establishment of chronic inflammation.

1.3. GRANULOMATOUS VASCULITIS AND CROHN'S DISEASE

1.3.1. Pathology of IBD

Crohn's disease. Crohn's disease can affect any part of the gastrointestinal tract from the mouth to the anus (although the terminal ileum is more commonly affected) and involves patchy inflammation of intestine (skip lesions; Whitehead, 1975). Crohn's disease, is characterised histologically by transmural inflammation, often involving granulomas, principally in a submucosal or serosal location (Jewell, 1990). Fissuring and fibrosis lead to complications such as fistula and stricture formation, often requiring surgical intervention.

Ulcerative colitis. Ulcerative colitis is a disease of the large bowel, involving the rectum and to a variable extent, the colon in a contiguous fashion. Ulcerative colitis, is characterised histologically by crypt abscesses, goblet cell depletion, and distorted microvillus architecture (Rhodes and Mayberry, 1990). Complications include toxic colonic dilation with perforation and a risk of colorectal neoplasia. There is some evidence to suggest that ulcerative colitis may have a vascular origin, as the extent of colitis has been shown to be dependent upon the territory of the mesenteric artery (Hamilton *et al.* 1995).

In both diseases, inflammatory cell infiltrates consist of lymphocytes, macrophages and plasma cells.

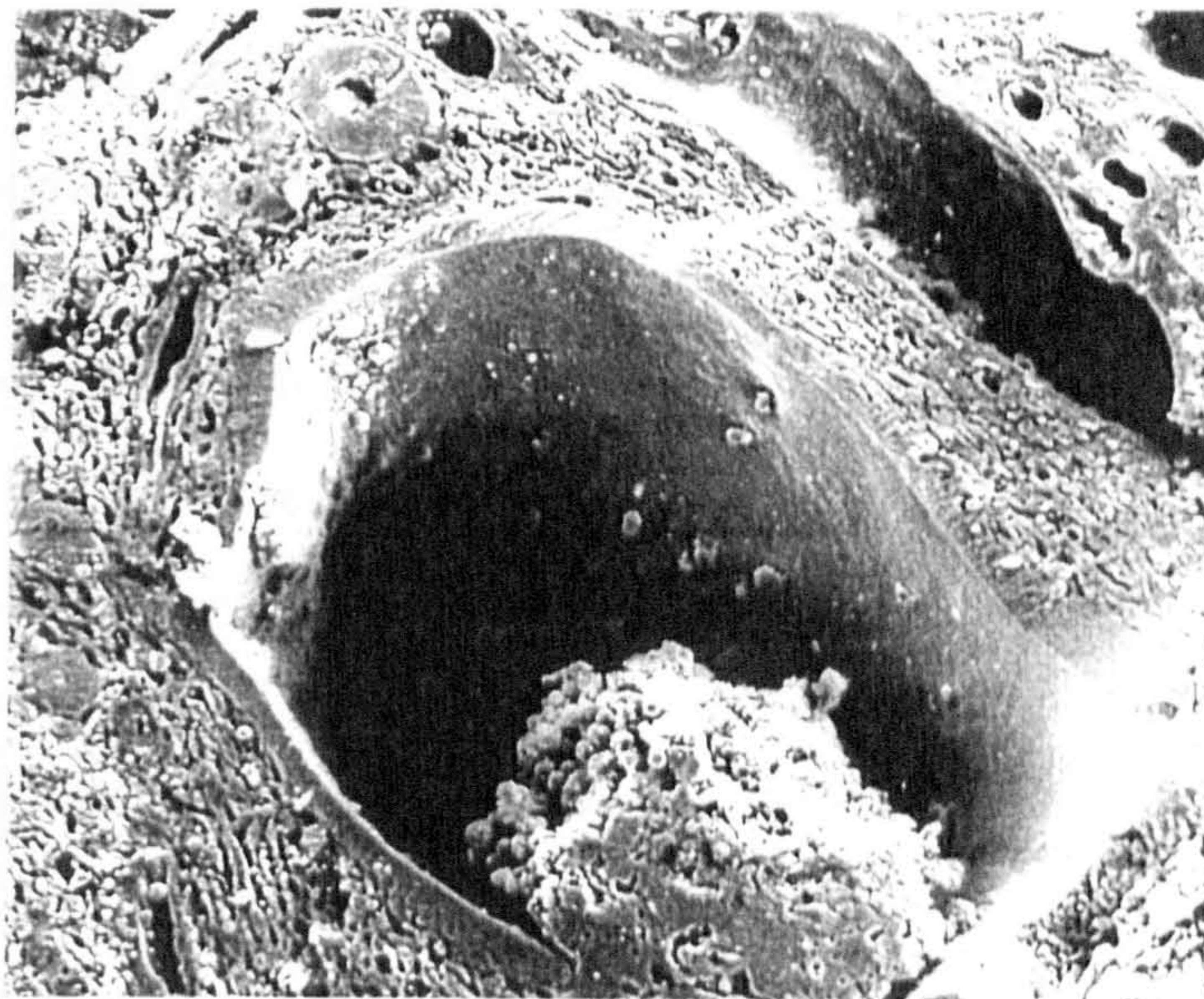
1.3.2. Granulomatous vasculitis and Crohn's disease

A study by Wakefield *et al.* (1989) set out to investigate the vascular lesion in Crohn's disease by examining perfusion-fixed resection tissue from 15 patients. The authors showed evidence for a sequence of events beginning with vascular injury and leading to fibrin deposition, vascular occlusion and neovascularisation. These data implied that vascular injury may be involved with the early pathogenesis of Crohn's disease.

Subsequent work by the same group used perfusion-fixed resection tissue to show that submucosal granulomas may be involved with vascular injury in the majority of tissues from patients with Crohn's disease (Wakefield *et al.* 1991a). A total of 485 granulomas were analysed in 24 perfusion-fixed resection

specimens of patients with Crohn's disease. A large proportion of these granulomas were found in the submucosa and the majority were identified as being involved with vascular injury. A subsequent study by Sankey *et al.* (1993) demonstrated that damage to the mucosal microvasculature occurred prior to infiltration of inflammatory cells, supporting the idea that vasculitis is an early feature of chronic intestinal ulceration rather than a secondary phenomenon.

Figure 1.4: Granulomatous vasculitis in Crohn's disease.



Electron micrograph of a perfusion-fixed sub mucosal blood vessel from a patient with Crohn's disease containing a granuloma. Original electron micrograph supplied by Dr A. Wakefield (personal communication).

Contrary to these data, Matson *et al.* (1995) identified only a minority of granulomas associated with blood vessels in patients with Crohn's disease. However, the tissue analysed in this study was not perfusion-fixed, and therefore the presence of granulomas occluding submucosal blood vessels may not have been recognised. Moreover, only a small number of local blood vessel-occluding granulomas may be required to cause widespread ischaemia, infarction and chronic inflammation.

The involvement of granulomatous vasculitis in Crohn's disease has been

supported by case reports (Motte *et al.* 1992; Weiss *et al.* 1995) and by experiments using an animal model of intestinal vasculitis to show that ferret mesenteric arteries injected with albumin microspheres develop submucosal vasculitis followed by discontinuous transmural inflammation (Osborne *et al.* 1993; Hudson *et al.* 1994).

Homeostatic changes. Evidence of active coagulation in blood vessels of Crohn's disease lesions has supported a role for granulomatous vasculitis in the early pathogenesis of Crohn's disease. A study at the Royal Free Hospital showed elevated concentrations of prothrombin fragments in patients with Crohn's disease at times of both active and inactive disease (Hudson *et al.* 1992), implicating thrombosis in the early stages of disease pathogenesis. Further work showed higher levels of markers for active coagulation (factor VII, lipoprotein a, fibrinogen) in the sera of patients with Crohn's disease compared with controls (Hudson *et al.* 1996). Additionally, low levels of factor XIIIa, the active subunit of factor VIII, in the sera of patients with active disease combined with strong immunostaining of mucosal microthrombi for factor XIIIa in the same patients, support the view that active coagulation is occurring in diseased tissue. Several independent studies by other research groups have also shown evidence for activation of coagulation in Crohn's disease (Chamouard *et al.* 1995; Smith *et al.* 1996; Chiarantini *et al.* 1996) and it has been shown that individuals with blood clotting abnormalities, such as haemophiliacs, are at a reduced risk of developing Crohn's disease (Thompson *et al.* 1995c).

Furthermore, epidemiological data shows that smoking and the oral contraceptive are risk factors for the development of Crohn's disease. The prothrombotic nature of these substances may exacerbate microvascular thrombosis in Crohn's disease

1.3.3. Conclusions

Crohn's disease is characterised histologically by granulomatous vasculitis and patients with Crohn's disease have increased plasma levels of prothrombotic risk factors and evidence of ongoing coagulation (Wakefield, 1995). As coagulation (leading to vasculitis and ischaemia) may play such an important role in the

early pathogenesis of Crohn's disease, it is likely that components of the intestinal submucosal microvasculature, for instance antigens expressed on microvascular endothelial cells, may lead to the aggregation of lymphocytes, leading to ischemia and producing discontinuous inflammation, ulceration and fibrosis. This is an attractive hypothesis since a vascular origin of Crohn's disease would explain the characteristic skip lesions observed.

1.4. BACTERIA AND CROHN'S DISEASE

There is little doubt that bacteria are involved with the pathogenesis of IBD: the possibility that a bacterial agent(s) is involved in the aetiology of IBD (in particular Crohn's disease) has received much attention ever since the first descriptions of the disease. The clinical benefits following metronidazole treatment in patients with Crohn's disease underlines the involvement of bacteria in the disease pathogenesis (Blichfeldt *et al.* 1978) as does the results of a recent study concerned with antibiotic therapy and Crohn's disease (Gui *et al.* 1997).

1.4.1. *Mycobacteria*

In 1913, Dalziel first recognised the similarities between Crohn's disease and Johnne's disease, a chronic granulomatous enterocolitis of cattle known to be caused by *Mycobacteria paratuberculosis* infection (Dalziel, 1913). Although no comparable amounts of *Mycobacteria* have been observed in Crohn's disease tissue, much work has been carried out into the possibility that a low copy number, persistent *Mycobacterial* infection may be involved with the aetiology of Crohn's disease (reviewed by Sanderson and Hermon-Taylor, 1992).

Different strains of *Mycobacteria* are often found in the lumen and tissues of healthy individuals but may be pathogenic in immunocompromised hosts (Hermon-Taylor *et al.* 1990). It is possible that individuals with an innate or acquired immune dysfunction could be susceptible to a persistent mycobacterial infection, leading to chronic granulomatous inflammation.

Bacterial culture. In 1984, van Kruiningen's group cultured a *Mycobacteria* (Chiodini *et al.* 1984a and b) which was later identified as *M. paratuberculosis* (Thayer, Jr. *et al.* 1984) from two patients with Crohn's disease, providing the first evidence for the presence of this organism in Crohn's disease. Subsequent work by the same group used infant goats injected with *M. paratuberculosis* isolated from a patient with Crohn's disease (van Kruiningen *et al.* 1986) to show the development of colitis without the microscopic presence of *M. paratuberculosis*. However, the numbers of IBD tissues positive for these *Mycobacteria* in culturing experiments have been too small to be of statistical

significance.

Culturing *Mycobacteria* from gut tissue requires several months and is prone to contamination from luminal bacteria not involved with disease pathogenesis. Moreover, the presence of *Mycobacteria* in ulcerated tissue may be due to an opportunistic secondary infection of diseased tissue, and therefore results from culturing experiments may be of little significance.

Serology. A study by Walmsley *et al.* (1996) analysed the sera of 40 patients with Crohn's disease and 61 control patients for anti-*M. paratuberculosis* IgA and IgG titres. No significant difference in antibody titres between Crohn's and control sera was observed and no correlation between antibody titre and disease activity was observed within the Crohn's disease group; results supported by a previous study (Stainsby *et al.* 1993). A subsequent immunohistochemical study of tissue from two French families with a high prevalence of Crohn's disease similarly failed to detect any association with *M. paratuberculosis* (van Kruiningen *et al.* 1993).

PCR. In an experimental model of gastrointestinal ulceration, Anthony *et al.* (1995) identified *Mycobacteria* within granulomatous of inflamed tissue, implicating *Mycobacteria* in the formation of granulomas.

The discovery and characterisation of the insertion sequence (IS) 900 by a group at St. George's Hospital (Green *et al.* 1989; Tizard *et al.* 1992) enabled PCR to be used for the detection of *M. paratuberculosis* DNA in IBD tissue. IS900 is a 1451 bp insertion element, highly specific to *M. paratuberculosis* and present at a copy number of 15-20 in the bacterial genome. Results from PCR experiments however, have been contradictory and inconclusive with numbers of *M. paratuberculosis* positive Crohn's disease samples ranging from 65% (Sanderson *et al.* 1992) to 0% (Rowbotham *et al.* 1995; Dumonceau *et al.* 1996). It remains possible that *M. paratuberculosis* is present in Crohn's disease tissue below the sensitivity limits of PCR and work is underway at St. George's Hospital to develop a PCR-based capture assay capable of detecting any *M. paratuberculosis* present in Crohn's disease tissue.

Taken together, culturing, serology and PCR studies so far suggest that

secondary infection by *Mycobacteria* may play an opportunistic role in the pathogenesis of IBD, but is unlikely to be involved with disease aetiology.

1.4.2. *Listeria*

Listeria monocytogenes has also been implicated in the aetiology of Crohn's disease after its identification in resection tissue (Liu *et al.* 1995). Seventy five percent of Crohn's disease tissues were positive throughout the inflamed mucosa for the bacteria using immunohistochemistry compared to 13% of ulcerative colitis tissues and none of the control tissues. However, significant numbers of tissues also gave positive immunohistochemical staining for *Escherichia coli* (*E.coli*; 57%) and *Streptococcus* (44%), indicating that the presence of *Listeria* may represent an opportunistic secondary invasion of diseased tissue rather than involvement in disease aetiology.

A recent immunohistochemical study has failed to find any association between *Listeria* infection and affected IBD tissue (Walmsley *et al.* 1997). Furthermore no molecular evidence of *Listeria monocytogenes* infection in Crohn's disease has been published, limiting the significance of the immunohistochemical results.

1.4.3. Conclusions

Several studies have provided evidence that an infectious agent(s) plays an important role in the aetiology of Crohn's disease. However, studies of *Mycobacteria*, *Listeria* and virulent *E. coli* strains in Crohn's disease tissue have given variable and contradictory results and it is likely that the presence of these organisms represent opportunistic secondary infections. Furthermore, antibiotic treatment of patients with IBD does not lead to clear-cut remission, suggesting that bacteria are involved with disease pathogenesis rather than aetiology.

Chapter 2

Chapter 2: CROHN'S DISEASE AND MEASLES VIRUS

2.1. BACKGROUND

2.1.1. Viruses and Crohn's disease

The possibility that Crohn's disease may have a viral aetiology is attractive as this would provide a relatively simple explanation for such a complex disease. Several groups have investigated such a possibility with varying success. In 1970, Mitchell and Rees injected homogenates of Crohn's disease tissue into footpads of immunodeficient mice, and reported the development of focal giant cell granulomas up to one year after inoculation. However, the formation of granulomas could have been due to the transfer into mice footpads of foreign material that was antigenically different to the host and pro-inflammatory cytokines.

This was followed in 1975 by an experiment by Aronson *et al.* who incubated homogenates of Crohn's disease tissue with cell cultures and observed a cytopathic effect characteristic of a viral infection. The next year, Gitnick *et al.* (1976) reported the isolation of viral agents from Crohn's disease tissue using a rabbit ileum tissue culture system and later putatively identified the virus as a picornavirus by electron microscopy (Gitnick and Rosen, 1976).

Later work, however showed that homogenates of control as well as Crohn's disease tissue elicited a cytopathic effect in cell culture (Yoshimura *et al.* 1984). In addition, no virus particles could be identified by electron microscopy and no virus could be passaged in cell culture suggesting that non-infectious factors were responsible for the cytopathic effect.

It is possible that in Crohn's disease tissue, very low numbers of viral particles are present which prevents their efficient isolation using cell and tissue culture methods. In addition, such a virus may have a defective replication by virtue of its persistence in diseased tissue, further inhibiting its efficient isolation and detection in cell culture. Such factors may explain the inconsistent reports of virus isolation from Crohn's disease tissue.

2.1.2. Measles virus

Epidemiological data supports an infectious aetiology for Crohn's disease (Ekbom *et al.* 1990; Ekbom *et al.* 1991; Gent *et al.* 1994). Measles, a paramyxovirus, has emerged as a candidate agent involved in the aetiology of Crohn's disease (Wakefield *et al.* 1993).

During acute measles infection, a range of tissues are infected including lymphoid tissue of the gut. Infection of lymphoid follicles may play a role in the development of viral persistence (Fournier *et al.* 1986), or in long term sequestration of viral antigens that may be responsible for the longevity of immunity to measles virus (Griffin *et al.* 1994). Persistent measles virus has been detected in a range of tissues and cell types (Brown *et al.* 1989) (including endothelial cells (Esolen *et al.* 1995)) many years after acute infection or vaccination (Kawashima *et al.* 1996b), and has been associated with the aetiology and pathogenesis of a number of chronic inflammatory diseases (Salmi *et al.* 1972; Norrby *et al.* 1974; Niedermeyer and Arnold, 1995; Schulz *et al.* 1992; Reddy *et al.* 1995; Reddy *et al.* 1996).

Occasionally, Koplik spots (a characteristic of acute measles) are observed in the gut (Carter and ter Meulen, 1990) which resemble, histologically, the aphthoid ulcers seen in Crohn's disease, and giant cell formation (a characteristic of measles virus infection *in vitro* and *in vivo*) is observed in foci of granulomatous inflammation in Crohn's disease (Wakefield *et al.* 1993).

The striking similarities between measles virus infection of the gut and the histopathological features of Crohn's disease were first reported in 1970 by Monif and Hood in a case of fatal measles with ileocolitis. The authors observed congested capillaries, extensive lymphocyte infiltration and lymphoid hyperplasia within infected intestinal tissue and argued that measles virus was responsible for the ileocolitis.

Persistent infection of endothelial cells with cytomegalovirus in the absence of cytopathology has been demonstrated (Smiley *et al.* 1988) and varicella-zoster virus infection of endothelial cells leading to vasculitis has been observed (Erhard *et al.* 1995). Measles virus has been shown capable of inducing vasculitis through infection of endothelial cells during acute infection (Carter and ter Meulen, 1990; Csonka *et al.* 1990). Expression of measles

haemagglutinin protein on the surface of infected cells has been shown to induce monocyte adhesion (Soilu-Hanninen *et al.* 1996) and *in vitro* studies by Mazure *et al.* (1994) have shown that infected endothelial cells express tissue factor, an important pro-coagulant. Persistent measles virus infection of endothelial cells has been observed in subacute sclerosing panencephalitis (SSPE; Kirk *et al.* 1991; Esolen *et al.* 1995; Isaacson *et al.* 1996), a disease caused by persistent measles infection. It has been postulated that infection of these cells may be responsible for the infiltration of inflammatory cells into infected tissue via monocyte adhesion (Cosby and Brankin, 1995).

Given these findings concerning viruses and endothelial cells, it is not unreasonable to postulate that measles virus may persistently infect a small number of intestinal microvascular endothelial cells for many years, eventually inducing a vasculitis leading to chronic intestinal inflammation. Measles is capable of infecting and causing ulceration throughout the gut but more specifically has tropism for lymphoid-rich tissue such as the terminal ileum - a commonly affected site of Crohn's disease.

2.2. EVIDENCE FOR AN ASSOCIATION BETWEEN MEASLES VIRUS AND CROHN'S DISEASE

Various epidemiological and immunological evidence has been accumulated by our group and others (some of which remains controversial), for and against an association between measles virus and Crohn's disease.

2.2.1. Epidemiology

A study of the Upsalla population in central Sweden found that more IBD births occurred during a three month period following measles epidemics compared with controls (Ekbom *et al.* 1994). This association was found to be statistically significant and suggests an association between early exposure to measles virus and the later development of IBD.

In contrast, Thompson *et al.* (1995b) analysed questionnaires concerning perinatal risk factors from almost 5,000 patients with IBD and controls in the UK. No birth-cohort effect for the development of IBD was seen in this group and no association between perinatal exposure to measles virus and subsequent development of IBD was observed. However, the same study (which elicited a response rate of only 20%) failed to show an association between exposure to measles virus and subsequent development of SSPE, a degenerative neurological disorder caused by a persistent measles virus infection, in which early exposure to measles virus has been shown to be a major risk factor (Clements and Cutts, 1995). Therefore, it could not be concluded from this study that early exposure to measles virus was not a risk factor for the later development of Crohn's disease.

Subhani *et al.* (1997) recently published results from a study of twins discordant for Crohn's disease, showing evidence that childhood measles infection (but not other childhood viral infections) is a significant risk factor for the development of IBD (relative risk = 2.1). In addition, work at the Royal Free Hospital (Montgomery, S.M., personal communication) has shown concurrent measles and mumps infections, below the age of seven, to be a significant risk factor for the development of Crohn's disease (relative risk = 9.1).

Ekbom *et al.* (1996) recently reported a study concerning Crohn's disease and in

utero measles virus exposure. Records of 25,000 pregnancies between 1940 and 1949 in Upsalla, Sweden, were studied and four cases of overt measles during pregnancy were identified. Three of the four children from these pregnancies were found to have developed Crohn's disease. Furthermore, the onset of Crohn's disease in each case was reportedly preceded by antibiotic-resistant pneumonia, suggestive of a viral infection. The only child not to develop Crohn's disease had typical measles at six years of age, indicating that although the mother was diagnosed with measles at the time of delivery, the baby was not infected with the virus and no immunity to measles developed.

It is important to note that the choice of time period for this study was based on two known cases of *in utero* measles virus infection followed by Crohn's disease during this time. Even allowing for this, the third case of *in utero* measles infection and Crohn's disease provides strong evidence for the involvement of perinatal measles virus exposure in the aetiology of Crohn's disease. Furthermore, immunogold electron microscopy has provided evidence of a persistent measles infection in tissue from these patients (Ekbom *et al.* 1996).

Contrary to these data, Jones *et al.* (1997) recently studied a cohort of over 3,000 individuals exposed to viruses *in utero*, including 47 exposed to measles. None of these individuals (aged 16-53) had progressed to develop Crohn's disease at the time of study.

In addition to this study, Nielsen *et al.* (1998) have also published negative findings concerning *in utero* exposure to measles virus and subsequent development of Crohn's disease. This study followed up 26 children of mothers admitted to hospital with measles during pregnancy. None of these children (36-79 years old at the time of study) subsequently developed IBD.

The lack of Crohn's disease cases in these studies raises the question as to whether the phenomenon of *in utero* exposure to measles virus and subsequent development of Crohn's disease is dependent on other factors. It is possible that the different populations studied were exposed to different strains of measles virus, accounting for the discrepancies in results between these studies. Additionally, infant mortality rates may be a confounding factor: in areas of high infant mortality, individuals infected with measles virus at an early age may not

have survived, whereas in areas of low infant mortality, individuals may have survived measles infection and later developed Crohn's disease. A study from the Royal Free Hospital has shown infant mortality rates to be inversely proportional to IBD incidence rates, supporting this possibility (Montgomery *et al.* 1997). The rate of infant mortality is also higher in the UK than in Sweden and Denmark, and this may contribute to the differences between the studies of Ekbohm *et al.* (1996), Jones *et al.* (1997) and Neilsen *et al.* (1998).

Measles vaccination. As discussed earlier, the incidence of IBD in children has increased ~~was~~ dramatically since the 1970's (Barton *et al.* 1996; Cosgrove *et al.* 1993). In view of this, Thompson *et al.* (1995a) decided ~~the~~ study the possibility of live, attenuated measles vaccine as a risk factor for the later development of IBD. This study determined the prevalence of coeliac disease, peptic ulceration, Crohn's disease and ulcerative colitis in over 3,500 individuals who had taken part in the Medical Research Council's 1964 trial of the live, attenuated measles vaccine. The control group for this study consisted of over 11,000 individuals from the National Child Development Study (NCDS), a longitudinal study of over 16,000 people born in the UK during a week in 1958 (11 years before the start of the national measles vaccination program). No significant associations were observed between coeliac disease or peptic ulcers and measles vaccination. However, the relative risk of measles vaccination was found to be 2.95 and 2.05 for the later development of Crohn's disease and ulcerative colitis respectively, indicating that measles vaccination may be a significant risk factor for the later development of IBD. h

The vaccine study group were born throughout the year while the NCDS group were born in one week of the year and therefore seasonal variations, in terms of birth dates of patients with IBD, may contribute to artifactual differences in the prevalence of IBD between these two groups. However, no studies have reported seasonality for IBD births in UK populations. ✓

Differences in the questionnaires sent to vaccine recipients and control groups may have led to an ascertainment bias, despite which, the rate of IBD prevalence in the control group was the highest reported rate in the world up to the time of the study (254 per 100,000). but they have 100,000 ! ?

The higher mean age of the NCDS group (33 years compared with 31 years) should have led to even more reported cases of IBD compared with the vaccine trial group. Therefore the increased risk of the development of IBD associated with measles vaccination is more likely to be a real phenomenon and not an artefact of the study method.

Contrary to these data, Hermon-Taylor *et al.* (1995) analysed the incidence of Crohn's disease in the UK and demonstrated that the incidence of Crohn's disease was increasing before the introduction of the measles vaccine in 1968. This indicates that measles vaccination may not have played a significant role in the observed increase in Crohn's disease incidence since the 1940s. Moreover, a recent study of a UK population (Feeney *et al.* 1997) has shown no association between measles vaccination and the later development of IBD in a study of 140 IBD patients and 280 controls. Although a different methodological approach was used, the population studied was similar to that of Thompson's study and would have received the same vaccine.

2.2.2. Electron Microscopy

In 1993, Wakefield *et al.*, using transmission electron microscopy, reported the presence of inclusion bodies containing paramyxovirus (the family containing measles virus) nucleocapsid-like particles at the foci of granulomas and in the submucosal endothelium associated with vascular injury in all 9 patients with Crohn's disease studied. Inclusion bodies are a feature of persistent paramyxovirus infection, consisting of condensations of viral nucleocapsids.

This work was supported by a subsequent independent electron microscopy study by Knibbs *et al.* (1993) who analysed diseased tissue from two French families with a high frequency of Crohn's disease for the presence of viral particles and described intranuclear inclusions, containing paramyxovirus-like particles, in submucosal vascular endothelial cells.

2.2.3. *In situ* hybridisation

In the same study, Wakefield *et al.* (1993) used *in situ* hybridisation, with an RNA probe complementary to measles nucleoprotein genomic RNA on Crohn's disease and control tissue. The probe hybridised to the cytoplasm of giant cells

within granulomas and to the nucleus of macrophage and lymphocytes within lymphoid follicles in all of 10 cases of Crohn's disease studied. Moreover, hybridisation signal also was observed within the submucosal microvasculature of 9 of 10 of these cases of Crohn's disease. Four out of 10 cases of ulcerative colitis and 3 out of 10 non-inflammatory control cases also gave hybridisation signal, suggesting that measles virus infection of intestinal tissue may not be a rare phenomenon.

Negative control experiments using probes complementary to vector sequences, Epstein-Barr sequences and cytomegalovirus sequences all gave negative staining. Moreover, ribonuclease digestion of tissue sections, prior to *in situ* hybridisation, prevented hybridisation signal using the measles-specific probe. Therefore, the hybridisation signals generated within Crohn's disease granulomas are unlikely to be artefactual.

2.2.4. Serology

Blazon *et al.* (1997) have recently reported raised serum measles IgM levels in patients with IBD using an indirect fluorescent antibody measles test system. Thirty six patients with Crohn's disease, 22 patients with ulcerative colitis and 89 control patients were tested for measles IgM. Seventy eight percent of Crohn's disease sera showed raised measles IgM levels, compared to 59% of ulcerative colitis sera and 3% of control sera and no correlation was found between total IgM levels and measles IgM levels. These results are suggestive of an ongoing immune response to measles in patients with IBD.

Support for these results has come from a recent Argentinean study of measles IgM levels in IBD patients and controls (Boerr *et al.* 1997). Twenty nine percent of Crohn's disease patients were found to have raised measles IgM levels, compared to 10% of ulcerative colitis patients and 3.7% of controls.

In contrast, Trouze *et al.* (1995) used an immunofluorescence assay to measure measles IgM levels in IBD and control patients. No significant difference was observed in between IBD and control samples with only one sample from each group giving a positive result. In addition, Fisher *et al.* (1997) found no significant difference between Crohn's disease sera and control sera in terms of anti measles IgG and measles IgM using a complement fixation test and ELISA

respectively. Lower levels of measles antibodies were observed in Crohn's disease sera which the authors argued may be indicative of a host immune defect.

It is possible that the different populations studied may account for these contradictory results.

2.2.5. Immunohistochemistry

Immunohistochemical studies have been performed by Wakefield *et al.* (1993), using a monoclonal antibody directed against measles nucleoprotein. Experiments showed positive staining in the foci of granulomatous inflammation in 13 of 15 patients with Crohn's disease. The monoclonal antibody used was shown to give positive staining in measles-infected neuronal cells of SSPE and measles inclusion body encephalitis (MIBE) tissue.

Subsequently, double staining experiments were performed at the Royal Free Hospital using an antibody against either a cytotoxic lymphocyte marker or a macrophage marker, together with a measles nucleoprotein polyclonal antibody, to determine the type of immune response directed against measles-infected cells in Crohn's disease granulomas (Wakefield *et al.* 1997). The measles polyclonal antibody sera used in this study was raised against an *E.coli* vector expressing recombinant measles nucleoprotein. As a negative control, polyclonal antibodies directed against *E.coli* did not give positive signal in the same tissue samples (unpublished data). Control tissue from patients with SSPE and measles appendicitis showed infected cells to be associated with cytotoxic lymphocytes while Crohn's disease granulomas showed infected cells to be associated with macrophages. These data are suggestive of an attenuated cytotoxic lymphocyte response in conjunction with an abnormal macrophage response to measles virus-infected cells in patients with Crohn's disease.

Support for these results has come from an independent study by Miyamoto *et al.* (1995) who identified a monoclonal antibody directed against the measles matrix protein which produced a positive signal in a significant number of Crohn's disease tissue specimens studied compared to ulcerative colitis and controls, using immunofluorescence. In addition, positive fluorescence was more commonly associated with granulomatous areas. Antibodies specific to

other measles virus antigens were negative, as were antibodies against herpes simplex virus type 1.

Contrary to these data, Liu *et al.* (1995) were unable to detect measles nucleoprotein in Crohn's disease tissue using a measles monoclonal antibody. Such a monoclonal antibody, recognising a single epitope on a measles antigen may lack the sensitivity of a polyclonal antibody and may not be able to detect low copy number measles antigens. Additionally, mutations involved with measles virus persistence may alter viral protein epitopes, allowing measles antigens to evade monoclonal antibody detection.

Liu *et al.* also used a measles nucleoprotein polyclonal antibody employed by Lewin *et al.* (1995), Ekblom *et al.* (1996) Wakefield *et al.* (1997) and Daszak *et al.* (1997), and described non-specific staining in a variety of cell types in tissue from Crohn's and control patients.

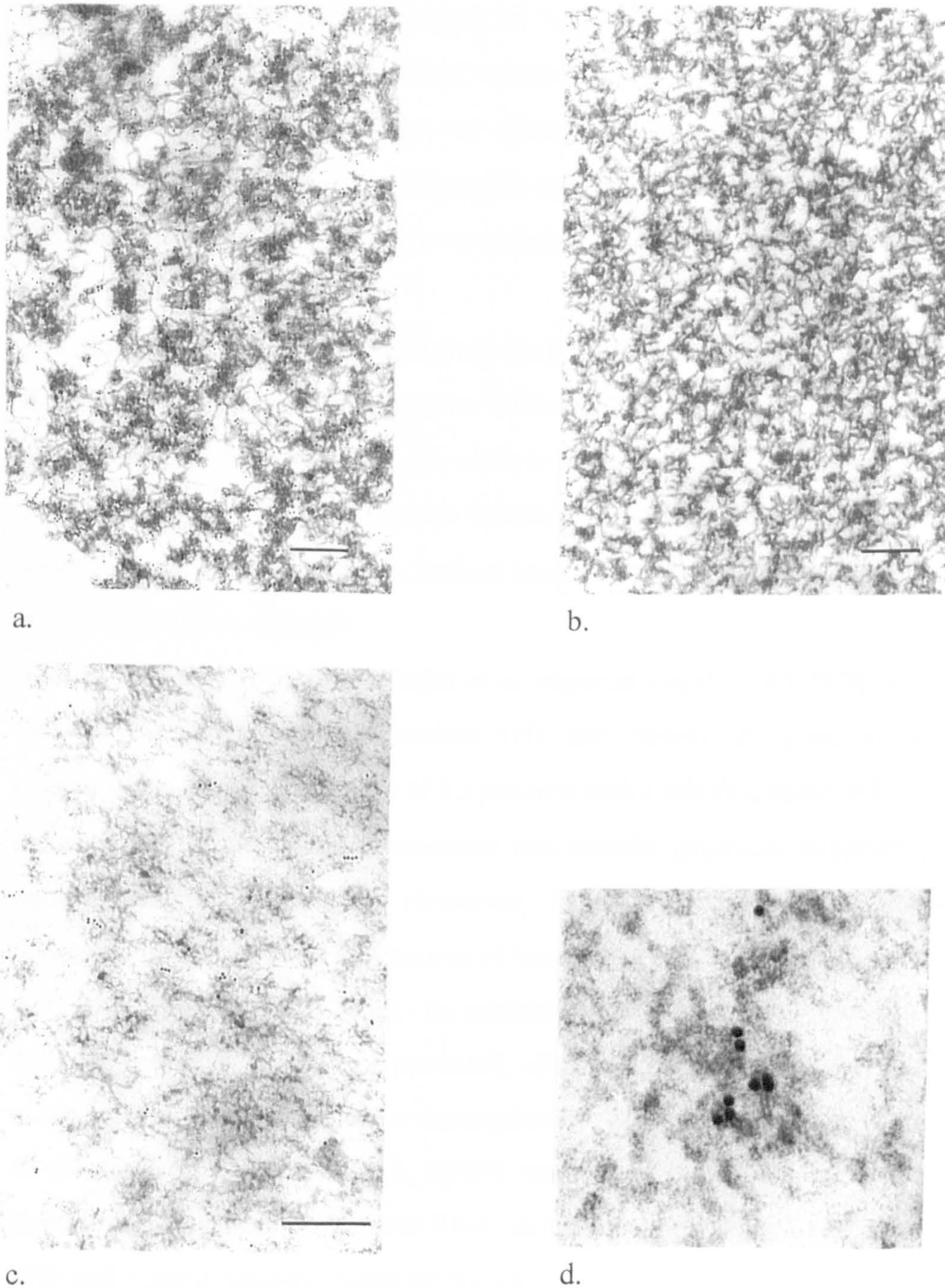
The discrepancy between the results of Liu *et al.* and Wakefield *et al.* in terms of polyclonal antibody staining may be explained by different methodologies, tissue samples, or interpretation of results. Work is underway at the Royal Free Hospital to reproduce positive immunohistochemistry results on Crohn's disease tissue using polyclonal antibody sera raised against measles nucleoprotein produced by an adenovirus vector.

2.2.6. Immunogold electron microscopy

The same polyclonal antibody sera used to generate staining in the granulomas of Crohn's disease tissues was labelled with gold particles for use in electron microscopy studies. This labelled antibody showed the predicted staining pattern in SSPE tissue at a resolution high enough to be able to show antibody binding in pairs along the length of viral nucleocapsids (Lewin *et al.* 1995). Six granulomatous Crohn's disease tissues and two ileocaecal tuberculosis tissues were examined for the presence of measles nucleoprotein using this technique. Five of the Crohn's disease samples and one of the ileocaecal tuberculosis samples showed positive staining in the foci of granulomatous inflammation. Moreover, as in SSPE tissue, pairs of antibodies binding to nucleocapsid-like particles were observed. The signal generated by this technique was very focal and indicated the presence of measles nucleocapsids at a much lower copy

number than that seen in SSPE tissue (Figure. 2.1). These results indicate the presence of a focal, low copy number persistent measles virus infection and may partly explain the reported inability to detect measles RNA in Crohn's disease tissue using reverse transcription-PCR as discussed below (Smith, 1994; Lewey *et al.* 1995; Iizuka *et al.* 1995; Fan *et al.* 1996; Haga *et al.* 1996).

Figure 2.1: Immunogold electron microscopy for measles nucleoprotein in Crohn's disease.



(a.) Electron micrograph of immunogold staining for measles nucleocapsids in SSPE tissue.

(b.) Electron micrograph of SSPE tissue section with no primary antibody as a negative control.

Low power (c.) and high power (d.) electron micrographs, receptively, of immunogold staining for measles nucleoprotein in a Crohn's disease granuloma. Data supplied by Dr A. Wakefield (personal communication).

2.2.7. Reverse transcription-PCR

Confirmation of the presence of measles RNA in IBD tissue, but not in control tissue, would provide strong evidence for a causal association between measles virus and IBD. RT-PCR is an ideal method for the detection of measles RNA due to its specificity and sensitivity (Bruce, 1993). Moreover, measles PCR products can be sequenced to determine the strain and origin of the virus, for instance if the virus originated from measles vaccine or a circulating wild-type virus (Rima *et al.* 1995).

In a study at the Royal Free Hospital, Smith (1994) reported negative measles N-gene RT-PCR results for measles virus RNA in IBD and control resection tissue. Positive PCR results were shown to have arisen as a result of PCR contamination in these experiments following sequence analysis. However, the sensitivity limit of the detection method used was not accurately determined and may not have been optimal.

In a letter to Lancet in 1995, Iizuka *et al.* reported negative RT-PCR results for measles matrix (M), haemagglutinin (H), and fusion (F) gene RNA from resection and biopsy specimens of 15 patients with Crohn's disease. RT-PCR for measles nucleoprotein RNA generated non-specific products, suggesting sub-optimal reaction conditions. Moreover, formalin-fixed, paraffin embedded resection tissue was used as a source of total RNA and efficient RNA isolation from this material is difficult. In addition, quantification of measles RNA detection sensitivity was not provided, allowing for the possibility that any measles RNA present was below the sensitivity limit of the detection assay.

In the same year, Lewey *et al.* (1995) reported results of an RT-PCR study directed against measles M-gene RNA using ileocolonic biopsy material from IBD and control patients. None of the samples gave positive results after RT-PCR although supernatant from measles infected cell culture, spiked into control biopsy sample yielded a positive result using the method described. Quantification of detection sensitivity was not provided in this study, allowing for the possibility that any measles RNA present may have been below the detection limit of this particular method.

In 1996, Haga *et al.* reported negative results from measles RT-PCR experiments using resection tissue from IBD and control patients. Measles N-

and F- gene RNA, in addition to mumps and rubella RNA, were used as targets for amplification. The authors claimed to be able to detect one measles genome in the samples tested, a claim which was based on the assumption that one measles viral genome is present per measles virion (one plaque forming unit of measles virus). However, Lund *et al.* (1984) have shown that approximately 1000 copies of the measles virus genome may be present in each measles virion. Moreover, more than one virion may be responsible for the formation of a plaque on a cell monolayer, therefore the measles RNA detection limit may have been several orders of magnitude less sensitive than that described. The authors also reported a long delay (between 20 and 90 minutes) between resection and freezing of tissue samples. This delay could lead to significant RNA degradation, particularly in the case of low copy number RNA species. In order to assess RNA integrity and detect significant RNA degradation, a low copy number RNA species should have been used as an internal positive control. Instead, a high copy number RNA species (β -actin mRNA) was used to detect RNA degradation. β -actin mRNA may have been detectable after degradation of low copy number RNA species and is therefore a poor indicator of RNA integrity. Therefore the results of this study do not preclude the possibility that measles RNA is present in IBD tissue below the detection sensitivity limit of the assay used.

Fan *et al.* (1996) have also used RT-PCR to search for measles RNA in IBD tissue. Biopsy and resection samples were obtained from IBD and control patients with 9 of the 10 Crohn's disease samples containing granulomas. The presence of Epstein-Barr virus DNA in approximately half of all samples and cytomegalovirus DNA in a minority of all samples did not represent a statistically significant difference. A combined RT-PCR reaction with *rTth* DNA polymerase, failed to detect measles N-gene RNA in any sample, although this method successfully amplified measles N-gene RNA in samples from measles vaccine recipients. As in previous studies to determine the presence of measles RNA in IBD tissue, the sensitivity of detection methods was not accurately quantified.

It is possible that measles antigens are present in the absence of detectable measles RNA. For instance, measles nucleoproteins may accumulate in the

nucleus of infected cells during persistent infection, and these may be present long after measles RNA has been degraded during chronic inflammation.

2.2.8. Conclusions

Epidemiological studies have provided strong circumstantial evidence for an association between early exposure to measles virus and the later development of IBD, in particular Crohn's disease. Some serological studies have shown raised measles IgM levels in IBD patients compared to controls. Electron microscopy studies have shown the presence of paramyxovirus nucleocapsid-like particles in Crohn's disease tissue. Studies to determine the presence of measles antigens in IBD tissue have yielded conflicting results although immunogold electron microscopy data suggest the presence of a persistent, low copy number measles virus infection restricted to the foci of granulomatous inflammation. *In situ* hybridisation experiments have supported these results, showing a very focal distribution of measles RNA.

However, the epidemiological, serological, and immunohistochemical data in favour of an association between measles virus and IBD have all been contradicted by various studies. One possible explanation for this is that exposure to measles virus may be an aetiological factor in a certain subgroup of IBD patients. Therefore, studies on different populations of IBD patients are likely to yield different results.

RT-PCR experiments from four different groups have not detected measles virus RNA in any IBD sample tested, however none of these studies have accurately quantified measles RNA detection sensitivity. In addition, some of these studies used an inappropriate target to assess RNA integrity (e.g. β -actin mRNA) rather than a low copy number housekeeping RNA species. The possibility of low copy number measles RNA present in these tissues, as suggested by other data, cannot be excluded. Difficulties in detecting measles N-gene RNA by RT-PCR may be responsible for negative results in some cases (Griffin, D.E., Johns Hopkins University, Baltimore; personal communication). These difficulties may arise due to mutations in the N-gene during persistent infection, preventing efficient amplification of N-gene RNA. Alternatively, strong binding of measles nucleoprotein to measles N-gene RNA as described by Andzhaparidze *et al.*

(1987) may prevent its efficient extraction leading, to false negative results. Also, measles nucleoprotein bound to N-gene RNA may prevent its efficient reverse transcription and amplification.

The evidence so far justifies further investigation to determine the presence of measles RNA in IBD tissue using molecular biology techniques.

2.3. MMR VACCINE-ASSOCIATED ILEO-COLONIC LYMPHOID HYPERPLASIA AND NON-SPECIFIC COLITIS IN CHILDREN WITH DEVELOPMENTAL DISORDER (AUTISTIC ENTEROPATHY)

An ongoing study at the Royal Free hospital has identified a group of children with ileo-colonic lymphoid hyperplasia, non-specific colitis and developmental disorder (Wakefield *et al.* 1998b). This new syndrome has been termed "autistic enteropathy" bearing a temporal association with the combined measles, mumps and rubella (MMR) vaccine introduced in 1988. Developmental disorder is similar to autism but differs in that patients develop normally for the first few years of life before degenerating to an autistic condition. Of twelve children studied in depth, all but one were male, reflecting the sex ratio seen in autism.

Onset of behavioural abnormalities varied between one day and 8 weeks from the time of vaccination. On endoscopic examination, lymph node hyperplasia was seen in the terminal ileum, in 10 of 12 patients. One further patient, in whom the terminal ileum was not intubated, was found to have had lymph node hyperplasia on barium follow through. Histological examination showed the presence of chronic, non-specific colitis in 11 of 12 patients, while nine age-matched control tissues were found to be histologically normal. It has been suggested that these patients may represent early cases of chronic IBD (Wakefield *et al.* 1998b).

Gut abnormalities have previously been reported in autistic children and have been associated with disease aetiology (D'Eufemia *et al.* 1996), and exposure to measles and rubella virus have been associated with the development of autism (Wing, 1996). In addition, persistent measles vaccine strain RNA has recently been detected in peripheral blood cells of patients with autoimmune hepatitis (Kawashima *et al.* 1996b), indicating that attenuated measles vaccine strain virus may be able to persist in some individuals.

2.3.1. Immunohistochemistry

Immunohistochemical studies were performed to determine which cells were involved with the inflammatory process in these cases and if there was any

evidence of viral involvement. Antibodies against CD68, a macrophage marker, and CD3 a pan T cell marker, showed a significant increase in the number of these cells in the submucosa and lamina propria of affected cases compared to controls. Immunohistochemistry for MHC class II antigen showed a marked increase in the number of antigen presenting cells compared with controls, including staining on endothelial cells, monocytes and epithelial cells in the cases studied. These results are indicative of chronic intestinal inflammation.

2.3.2. Evidence for the presence of measles virus

Immunohistochemistry for measles nucleoprotein was performed using polyclonal antibody sera (RAd 68) from mice raised against recombinant measles nucleoprotein purified from an adenovirus expression system (Wakefield *et al.* 1998a). Staining was shown in 5 out of 10 cases studied in lymphoid follicles and double staining with an antibody against CD21 (a dendritic cell marker) showed co-localisation of measles nucleoprotein staining with discrete follicular dendritic cells. Only 1 of 10 control biopsies stained positively for measles nucleoprotein.

RAd 68 showed positive staining in adenovirus-infected cells, indicating the presence of adenovirus-specific antibodies in the measles polyclonal sera. As a negative control, no staining was observed for adenovirus antigens in any intestinal tissue, indicating that adenovirus-specific antibodies present in RAd 68 were not responsible for the reported positive measles nucleoprotein staining. Moreover, preimmune sera from the measles virus nucleoprotein-immunised mouse gave negative staining in all tissues. However, a measles nucleoprotein monoclonal antibody failed to show any staining on any of these tissues. This may be due to an absence of measles monoclonal antibody recognition of mutated measles antigens, or low measles monoclonal antibody sensitivity.

Viral infection of dendritic cells. Follicular dendritic cells have been shown to be a reservoir for human immunodeficiency virus in AIDS patients leading to lymph node hyperplasia (Tacchetti *et al.* 1997). In these cells, HIV escapes recognition by cytotoxic T lymphocytes, enabling the virus to persist and infect CD4+ T helper cells. In addition, follicular dendritic cells may retain viral

antigen for long term memory, although evidence for this remains controversial (Doherty *et al.* 1996).

It has been demonstrated that dendritic cells of the central nervous system become infected during persistent measles virus persistence in SSPE (Allen *et al.* 1996). Recently, it has been shown that infection of dendritic cells infected with wild-type or vaccine strain measles virus reduces IL-12 production (Grosjean *et al.* 1997). Reduced IL-12 production may be responsible for disrupting the homeostasis of the gut, giving rise to chronic intestinal inflammation.

2.3.3. Conclusions

This group of paediatric patients may represent individuals with early onset inflammatory bowel disease. The temporal association with MMR vaccination and the initial immunohistochemical results, indicating the presence of measles virus in follicular dendritic cells, justify a molecular biology study to determine the presence of measles, mumps or rubella in clinical samples from these patients.

Chapter 3

Chapter 3. MEASLES VIRUS

3.1. ACUTE MEASLES INFECTION

Measles is an RNA virus of the genus morbillivirus within the paramyxoviridae family, containing a 15,898 base single-stranded genome of negative polarity. Other morbilliviruses include canine distemper virus and rinderpest virus. First isolated in 1954 by Enders and Peebles, measles virus infection has since been shown to be restricted to higher primates and is responsible for over one million deaths per year world-wide.

3.1.1. Sites of infection

Measles is a highly infectious virus which enters the host via the upper respiratory tract, infecting local lymph nodes (Carter and ter Meulen, 1990). The primary viremia consists of virus spread and replication in the rest of the respiratory tract and the reticuloendothelial system via the blood. This is followed several days later by the secondary viremia, consisting of infection of endothelial and epithelial cells via infected circulating leukocytes. Multinucleated giant cells (also known as Warthin-Finkeldy cells) containing inclusion bodies, form in all infected tissues (including the gastrointestinal tract; Tokunaga *et al.* 1980; Monif and Hood, 1970) and lymphoid hyperplasia occurs in infected organs, associated with inflammatory cell infiltrates (Carter and ter Meulen, 1990). Infection of the gastrointestinal tract can lead to measles enteritis in some individuals (Tamboen *et al.* 1991).

Following the secondary viremia, virus shedding occurs in sputum, nasopharyngeal secretions and urine. Koplik spots, small aphthoid ulcers characteristic of acute measles, develop in buccal mucosa and conjunctivae (Carter and ter Meulen, 1990). This is followed by the appearance of a maculopapular rash spreading over the entire body. Complications of acute measles include giant cell pneumonia and autoimmune encephalomyelitis, resulting from a host immune response to myelin basic protein in the CNS in the apparent absence of measles virus (Carter and ter Meulen, 1990).

3.1.2. Immunology

During the rash (approximately 16 days after initial infection) measles IgM is produced followed by measles IgG (Schluederberg, 1965). The major antibody response is directed against measles nucleoprotein, while antibodies against measles haemagglutinin protein are the major neutralising antibodies, preventing further viral infection, clearing the virus from the blood and lysing infected cells (Norby and Gollmar, 1972). There is some evidence to suggest that this response clears infectious virus particles, allowing defective virus particles to establish a persistent infection (Rammohan *et al.* 1981).

The cell-mediated immune response plays a major role in clearance of measles virus from infected tissues. CD8⁺ cytotoxic T cells, recognising measles virus antigens associated with MHC class I molecules, are detectable in the blood during acute infection and are responsible for lysis of measles-infected cells (Griffin *et al.* 1989). CD4⁺ T cells, recognising measles antigens associated with MHC class II molecules are also responsible for lysis of infected cells in addition to secreting a range of cytokines involved with the recruitment of monocytes and the regulation of B and T cell differentiation (van Binnendijk *et al.* 1990).

It has been shown that prior to the appearance of the rash, Th1 cells are activated, while following the rash, Th2 cells (producing IL-4) are activated (Griffin and Ward, 1993).

IL-4 is involved with B cell activation and levels of serum IL-4 remain elevated for several weeks following acute infection (Ward and Griffin, 1993). Reduced Th1 cell activation results in poor cell-mediated immune response and is responsible for the observed immunosuppression following acute infection, leading to opportunistic secondary infections responsible for much of the morbidity and mortality associated with acute measles (Burstrom *et al.* 1995).

Longevity of measles immunity. The longevity of the antibody response to measles virus has led some researchers to suggest that persistence of some component of the virus may be essential in stimulating lymphocytes and maintaining measles adequate antibody titres (Griffin *et al.* 1994). Indeed, a sub-optimal cell-mediated immune response during the later stages of infection may

aid the establishment of persistent measles virus infection in lymphoid tissues which may be involved with this longevity of immunity to measles. Although there is little evidence of widespread persistence of infectious measles virus in an immune population, it is possible that a defective form of measles virus or measles antigen may persist in lymphoid tissue which is not readily detected using molecular or cell culture methods. However, the role of antigen retention in long-term immunity as proposed by Mandel *et al.* (1980) based on earlier work by Tew and Mandel (1979) remains controversial (Doherty *et al.* 1996). Such a mechanism would however, offer an explanation as to the discrepancy between negative RT-PCR results (Smith, 1994; Fan *et al.* 1996; Lewey *et al.* 1995; Iizuka *et al.* 1995; Haga *et al.* 1996) and positive immunohistochemical results (Miyamoto *et al.* 1995; Wakefield *et al.* 1993; Lewin *et al.* 1995; Daszak *et al.* (1997); Wakefield *et al.* 1997) for the presence of measles virus in IBD tissue.

3.1.3. Diseases associated with measles

Measles inclusion body encephalitis (MIBE). First described in 1977, MIBE is a rare, fatal neurological disorder caused by a defective measles virus infection of the central nervous system (CNS) in some immunosuppressed individuals following acute measles (Alcardi *et al.* 1977).

Subacute sclerosing panencephalitis (SSPE). SSPE, another rare, fatal neurological disease, was proposed to be caused by a persistent measles virus infection after the detection of measles antigens in diseased tissue by immunofluorescence (Legg, 1967). Raised measles antibody levels in serum and cerebrospinal fluid of SSPE patients were observed (Connolly *et al.* 1971) and subsequently, electron microscopy (Tellez-Nagel *et al.* 1967), *in situ* hybridisation (McQuaid *et al.* 1993) and more recently, RT-PCR studies (Godec *et al.* 1990) confirmed the presence of a persistent measles virus infection of the CNS, responsible for chronic inflammation and demyelination in affected individuals.

Autoimmune Hepatitis (AIH). AIH has also been associated with persistent

measles virus infection since Christie *et al.* reported elevated measles antibodies in the sera of patients with chronic hepatitis in 1983. Later Robertson *et al.* (1987) identified measles N-gene RNA in the peripheral blood mononuclear cells (PBMCs) of 12 of 18 AIH patients and these results have recently been supported by Kawashima *et al.* (1996b) who demonstrated the presence of measles H-gene RNA in the PBMCs of patients with AIH using RT-PCR. In the latter study, sequence analysis of PCR products identified vaccine strain measles virus in all four AIH paediatric patients studied, and wild-type measles RNA identified in the two adult AIH patients studied. Moreover, vaccine strain measles was detected in paediatric patients up to 11 years following vaccination, indicating that live attenuated measles vaccine may persist in peripheral blood for many years.

Otosclerosis. Recently, measles RNA has been shown to be present in diseased tissue from patients with otosclerosis a chronic inflammation of the otospongiotic area. McKenna *et al.* (1986) demonstrated the presence of paramyxovirus nucleocapsid-like structures in otosclerotic lesions using electron microscopy and measles antigens were subsequently identified in the foci of active disease using immunohistochemistry (McKenna and Mills, 1989). This work has been supported by recent data from Niedermeyer and Arnold (1995) who have detected measles RNA using RT-PCR in diseased bone tissue of 13 out of 14 cases of otosclerosis.

Paget's Disease. Nucleocapsid-like structures have been identified using electron microscopy in bone tissue from patients with Paget's disease (Mills and Singer, 1976) a chronic proliferative disease of bone osteoclasts. Subsequently, *in situ* hybridisation was used to detect measles RNA in diseased tissue (Basle *et al.* 1986) and measles RNA has been detected in PBMCs (Reddy *et al.* 1996) and diseased tissue (Reddy *et al.* 1995) of patients using RT-PCR. However, other studies have reported the presence of canine distemper virus in tissue from these patients (Cartwright *et al.* 1993; Gordon *et al.* 1992) and it is possible that co-infection with both measles and canine distemper virus may lead to the development of Paget's disease.

M.S. ref.

Others. Measles virus has been proposed to be involved with the aetiology of Kawasaki disease (Bansil *et al.* 1997; Dhib-Jalbut *et al.* 1990), a systemic vasculitis in young children, and multiple sclerosis (Schulz *et al.* 1992) although evidence for these associations remains controversial (Godec *et al.* 1992).

K.D

3.1.4. Conclusions

The detection of measles virus in conditions other than acute measles indicates that wild-type or vaccine strain measles virus can persist in some individuals for many years, causing chronic disease in a variety of tissue types. In most cases where an association has been shown between disease development and presence of measles virus, electron microscopy and immunology studies have been followed by RT-PCR studies to confirm the presence of measles virus in diseased tissue.

3.2. MEASLES VIRUS STRUCTURE AND REPLICATION

3.2.1. Transcription

It is thought that measles virus replicates in the same manner as other paramyxoviruses such as Newcastle disease virus (Hamaguchi *et al.* 1983), via an RNA-dependent RNA polymerase encoded by the measles phosphoprotein and large protein genes. This polymerase has no proof-reading activity, preventing the correction of errors in the nucleic acid sequence generated during virus replication, and accounting for the sequence variation between measles strains (Rima *et al.* 1995). It has been proposed that this lack of proof-reading ability is involved in the development of measles virus persistence in SSPE (Schneider-Schaulies *et al.* 1995).

The viral polymerase binds to a leader sequence at the 3' end of the negative strand measles genome (containing the N-gene) and sequentially transcribes the individual measles virus genes towards the 5' end (containing the large protein gene). During transcription, the polymerase detaches from the genomic RNA at intergenic regions following polyadenylation of mRNA and then reattaches to transcribe the next gene (Horikami and Moyer, 1995). In this manner a gradient of viral mRNA is produced from N- to L-gene mRNA (Cattaneo *et al.* 1987, Sidhu *et al.* 1994).

As is the case for vesicular stomatitis virus (Blumberg *et al.* 1981), the synthesis of high levels of measles nucleoprotein is thought to trigger a switch from transcription of viral mRNA to transcription of full length antigenomic RNA, for transcription back to negative genomic RNA. In this manner, no measles DNA is produced at any stage of the replication cycle. A diagram of a measles virion is shown in Figure 3.1. and a schematic diagram of measles virus replicative cycle is given in Figure 3.2.

3.2.2. Encapsidation

Genomic and antigenomic RNA molecules are encapsulated by measles nucleoproteins (N), forming tubular nucleocapsid structures which can be identified using electron microscopy (Nakai *et al.* 1969) and condensation of nucleocapsids leads to the formation of characteristic inclusion bodies in the

nucleus and cytoplasm of infected cells. Measles RNA polymerases associate with nucleocapsids during virion assembly (Huber *et al.* 1991), enabling active transcription to occur immediately on virus entry into host cells.

Measles matrix protein is thought to be involved with virion maturation, promoting the association between nucleocapsids (Hirano *et al.* 1992) and envelope proteins on the cell membrane and leading to virus budding (Peebles, 1991). Host cell-derived actin molecules have been shown to be associated with functional virions and these may also be involved with virus maturation (Tyrrell and Norrby, 1978). Mature virions may contain up to 1000 nucleocapsids, each containing a full-length molecule of genomic or antigenomic RNA (Lund *et al.* 1984).

Figure 3.1: Measles virus structure.

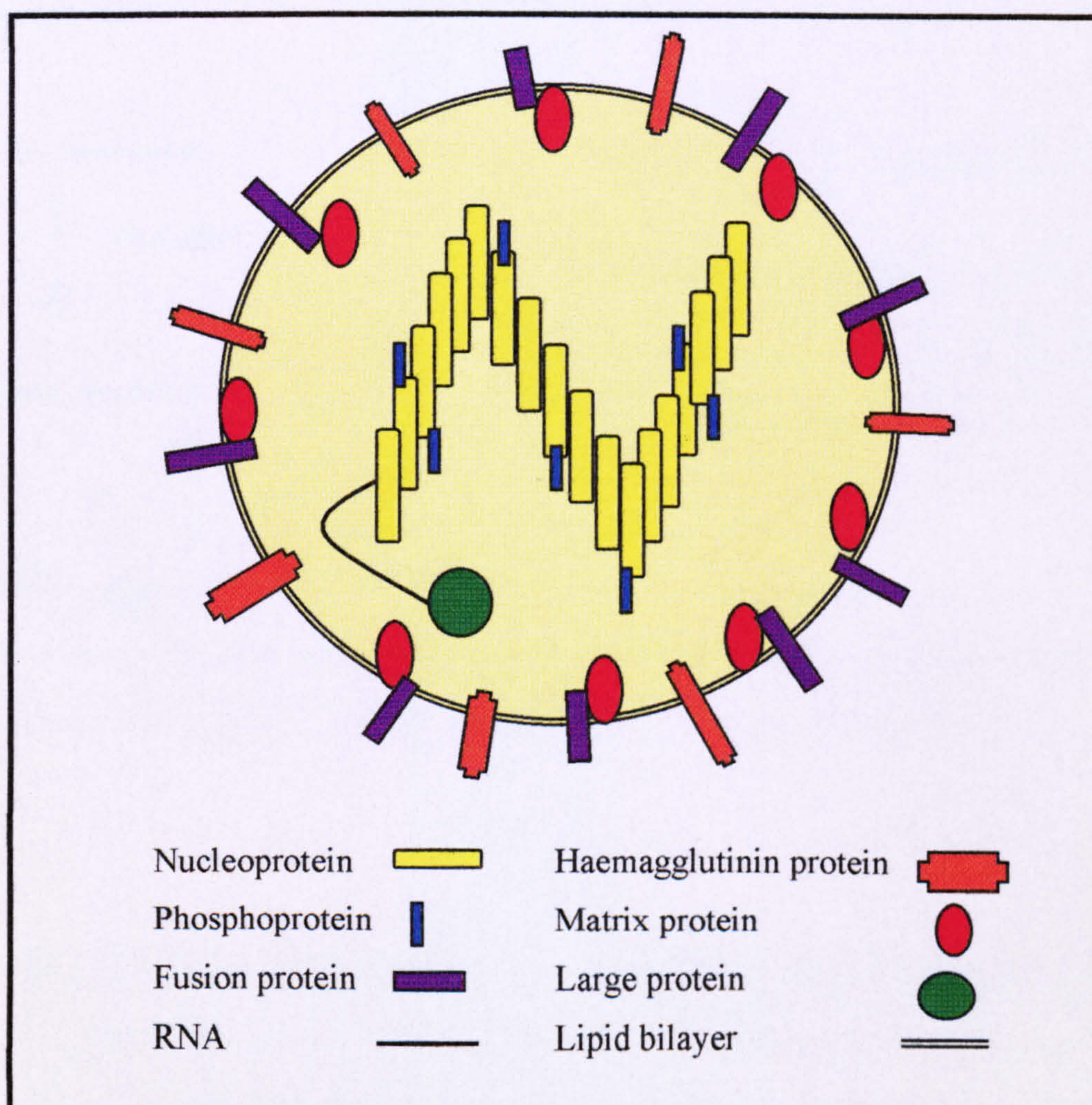
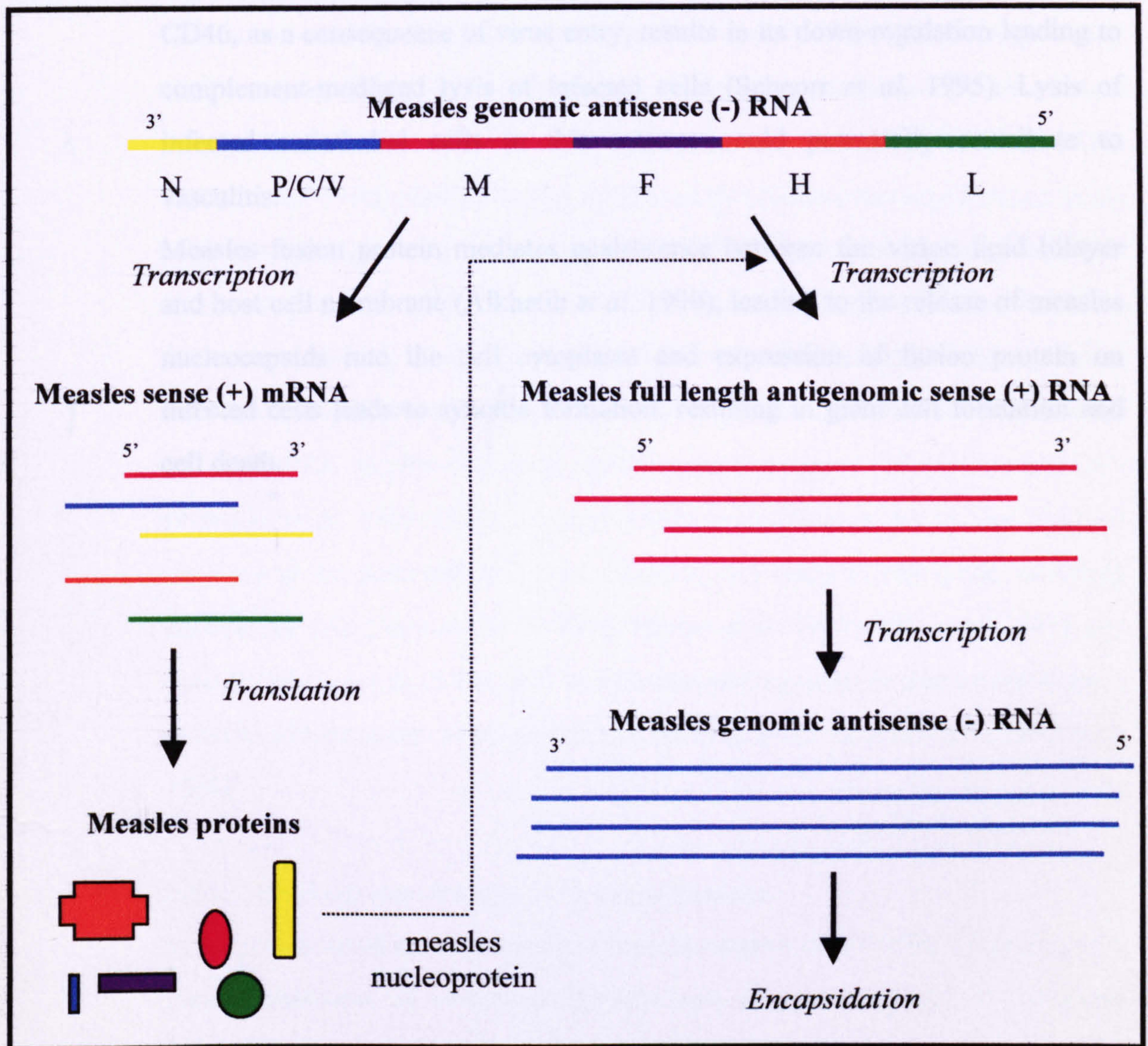


Figure 3.2: Measles virus replication.



Schematic representation of the measles replication cycle. Full-length genomic measles RNA is transcribed into mRNA then translated to viral proteins. A critical level of measles nucleoprotein is thought to be necessary to switch transcription to full-length antigenomic RNA which is subsequently transcribed back to full-length genomic measles RNA.

3.2.3. Virus entry

Entry to the host cell requires binding of measles haemagglutinin protein to its receptor, CD46 (Naniche *et al.* 1993) - a glycoprotein involved with down-regulating the complement cascade (Hourcade *et al.* 1989). Internalisation of CD46, as a consequence of virus entry, results in its down-regulation leading to complement-mediated lysis of infected cells (Schnorr *et al.* 1995). Lysis of infected endothelial cells in this manner could potentially contribute to vasculitis.

Measles fusion protein mediates coalescence between the virion lipid bilayer and host cell membrane (Alkhatib *et al.* 1990), leading to the release of measles nucleocapsids into the cell cytoplasm and expression of fusion protein on infected cells leads to syncytia formation, resulting in giant cell formation and cell death.

3.3. SUBACUTE SCLEROSING PANENCEPHALITIS

Persistent measles virus infection of the CNS is known to be the cause of SSPE. The risk of developing SSPE after acute measles infection and following measles vaccination, has been reported to be 4 and 0.14 in 100,000, respectively (Miller *et al.* 1992). Early exposure to measles virus (acute or sub-clinical infection) has been identified as a risk factor for the development of SSPE (Miller *et al.* 1992) and the first symptoms of this disease can appear many years after virus infection. Measles antigens and RNA have been detected in SSPE tissue (Godec *et al.* 1990; Legg, 1967); however replicating virus particles have never been directly isolated from SSPE tissues, indicative of defective viral replication (Carter and ter Meulen, 1990). High measles antibodies titres have been detected in the sera and cerebrospinal fluid of affected individuals (Freeman *et al.* 1967; Connolly *et al.* 1967), suggesting an active host immune response to the persistent infection. Infection of a range of cell types, including endothelial cells (Isaacson *et al.* 1996; Esolen *et al.* 1995; Kirk *et al.* 1991), has been shown to occur in the CNS and disease pathogenesis is characterised by a massive inflammatory cell infiltrate and demyelination of neuronal cells (Poser, 1990). b/

3.3.1. Mechanisms of measles virus persistence

No single strain of measles virus has been associated with SSPE, suggesting that the establishment of viral persistence occurs as a consequence of mutations following virus replication within the host. There are several possible mechanisms for the development of measles virus persistence in SSPE, which have been the subject of intense study as persistent measles virus infections may be involved with the aetiologies of a range of chronic diseases (Katz, 1995).

It is thought that virus enters the CNS during acute infection and the establishment of viral persistence follows in some individuals (Billeter and Cattaneo, 1991). Measles virus antigens and RNA have been detected in brain endothelial cells of children following acute measles (Esolen *et al.* 1995) indicating that infection of the CNS during acute measles may not be uncommon. Measles RNA sequences have also been detected in autopsied brain

tissue of patients without SSPE (Esolen *et al.* 1995), suggesting that measles persistence in the CNS may be more common than previously thought and this persistence may play a role in the longevity of immunity to further measles infection.

Persistence may also occur in other parts of the body prior to the establishment of persistence in the CNS (Schneider-Schaulies *et al.* 1991; Brown *et al.* 1989). Interestingly, Fournier *et al.* (1986) have detected measles RNA in lymphoid tissue of the appendix prior to the development of SSPE, raising the possibility that gut lymphoid tissue may serve as a reservoir for persistent measles replication.

In SSPE, a period of latent, subclinical infection of lymphoid tissue, blood leukocytes or neuronal cells, lasting several years is followed by a period of abnormal virus replication in neuronal cells which leads to the production of a range of cytokines, such as IL-2, IL-6 and IFN- β (Nagano *et al.* 1994), eliciting a chronic inflammatory response.

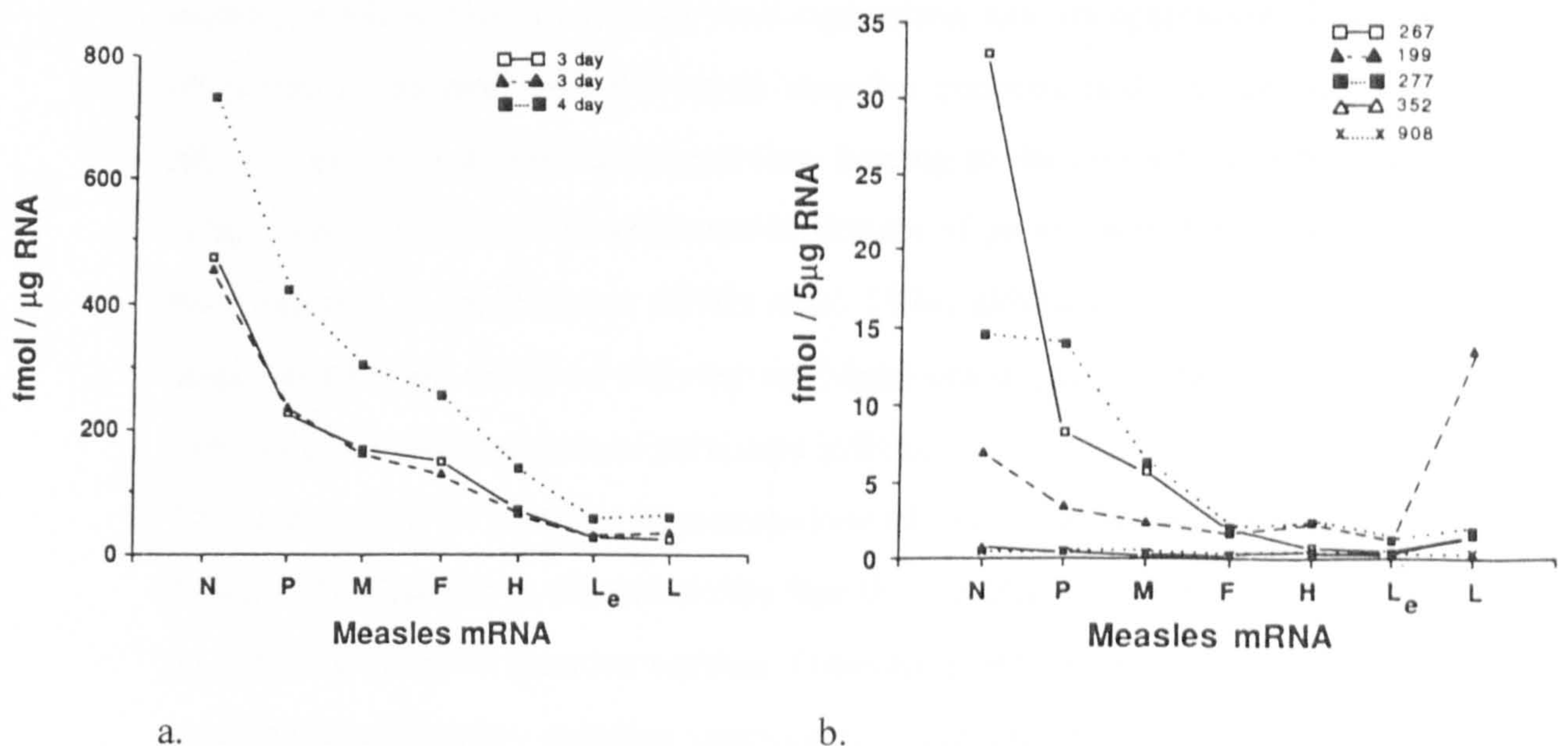
Mutations. A lack of virus polymerase proof-reading activity, together with a host cell double-stranded RNA unwinding/modifying enzyme activity, causes a high mutation rate during measles virus replication (Cattaneo *et al.* 1989). The host immune response recognises and lyses infected cells which express viral antigens on their surface, therefore mutations which prevent or decrease the expression of viral antigens on the host cell surface favour unchallenged viral replication. Such mutations can occur in the measles M-gene (Hirano *et al.* 1992; Hirano *et al.* 1993), preventing efficient virus maturation, or in the cytoplasmic tail region of the measles F-gene (Schmid *et al.* 1992), preventing efficient virus budding. In addition, mutations in the regulatory sequences of these genes may lead to down-regulation of their expression (Schneider-Schaulies *et al.* 1989; Hummel *et al.* 1994), leading to reduced virus maturation and budding.

In this manner, mutated measles virus can escape host immune detection for many years. During this period, mutations accumulate in genes not essential for viral persistence, such as the envelope genes (H and F) and the M-gene, causing their reduced expression, with no deleterious effect to viral RNA replication.

This accounts for the steep measles mRNA transcription gradient observed in SSPE tissue (Sidhu *et al.* 1994) since H, F and M genes are towards the distal 5' end of the viral genome (Figure. 3.3.). Sequencing of SSPE-derived measles RNA reveals the high level of mutations which makes the determination of the original persistence-inducing mutation(s) difficult.

Without efficient virus maturation or budding, measles nucleocapsids accumulate in the nucleus and cytoplasm of infected cells and spread throughout the CNS via cell-cell interactions (Cattaneo and Rose, 1993).

Figure 3.3: Measles virus transcription gradient.



The characteristic 3'-5' measles RNA transcription gradients generated in cells acutely infected with virus (a.), and cells persistently infected with virus (b.). This data was taken from a paper by Sidhu *et al.* (1994) with permission from Academic Press Inc.

Defective interfering particles. Another proposed mechanism of the development of measles virus persistence is the generation of defective interfering (DI) particles. Also known as 5' copy-back sequences, these particles

result from the aborted transcription of full-length measles antigenomic RNA to negative-strand genomic RNA (Sidhu *et al.* 1994; DePolo *et al.* 1987). Binding of the virus polymerase to a transcription promoter site at the 5' end of genome (containing the L-gene) promotes the transcription of genomic RNA during viral replication. However, transcription is occasionally aborted approximately 100 nucleotides downstream, leading to the formation of a hairpin-loop structure within this short transcript. The viral polymerase then binds to an internal pseudo-transcription promoter site, synthesising a second RNA strand. In this manner a DI particle is generated, consisting of a double stranded RNA region and a hairpin loop representing the terminal 5' end of the measles virus genome (Sidhu *et al.* 1994).

These DI particles contain transcription promoter sites and binding sites for measles nucleoproteins enabling their replication and encapsulation. Therefore DI particles compete with full-length measles genomic and antigenomic RNA for viral polymerase and nucleoproteins, leading to the restriction of functional virus replication and the possible establishment of persistence. DI particles have been detected in SSPE tissue (Sidhu *et al.* 1994) although it is unclear whether these particles are involved with the establishment of measles persistence or are generated as a consequence of persistent infection.

The detection of DI particles in preparations of live measles vaccine (Calain and Roux, 1988) has led to the possibility that these particles may be involved with the attenuation of the measles vaccine. Generation of these particles may provide a mechanism whereby measles vaccination could lead to viral persistence and the generation of lifelong immunity associated with measles.

3.4. MEASLES VACCINATION

Measles vaccination using a live, attenuated form of measles virus (introduced in the UK in 1968) has virtually eradicated the disease and its associated morbidity and mortality in developed countries (Clements and Cutts, 1995), although outbreaks of measles do occur in susceptible populations (Outlaw and Pringle, 1995).

Although vaccination programs have been successful in developed countries, measles virus has not been eradicated due to incomplete vaccination of populations in developing countries (Norrby, 1995) and world-wide, over one million people die each year from measles-associated illness.

In 1988 single measles vaccination was replaced by a combined preparation of live, attenuated measles, mumps and rubella (MMR) viruses and this has now been augmented by a two-dose vaccine schedule. However, adverse effects of MMR vaccination have been reported (Bennett *et al.* 1994; Miller *et al.* 1993), and persistent vaccine-strain measles virus has been detected in the liver (Kawashima *et al.* 1996b) and CNS (Matsuzono *et al.* 1995) associated with disease processes. In addition, cases of SSPE have been reported in individuals following measles vaccination (Miller *et al.* 1992).

3.4.1. Lifelong immunity

Circulating maternal measles antibodies confer protection to early acute measles infection (Norrby, 1995). However, prior to exposure to wild measles or measles vaccine, all individuals are susceptible to infection. Measles infection is known to confer lifelong immunity in all but immunosuppressed individuals without the need for re-exposure to the virus (Griffin *et al.* 1994). The mechanism for the longevity of immunity to measles is unknown although there is little evidence of widespread measles viral persistence in the general population.

It has been postulated that follicular dendritic cells may trap immune complexes containing measles antigens for many years, stimulating memory B-cells (Griffin *et al.* 1994) and it is possible that these cells, expressing measles antigens, could stimulate a chronic inflammatory response in some individuals with an immune dysfunction.

3.4.2. Vaccine preparation

Initial vaccine trials in the 1960s using inactivated measles virus gave rise to adverse side effects and incomplete immunisation leading to the development of a live attenuated measles vaccine (Enders *et al.* 1960). The Schwarz strain of measles vaccine is used in the UK, derived from the Edmonston B strain as are most measles vaccines used throughout the world.

Adapted to replication in chick embryos by repeated passaging, measles virus vaccine strains replicate poorly in human tissue, theoretically, allowing clearance of the virus by the host immune system before the development of acute disease. Although the exact mechanism of measles virus attenuation is unknown, it is likely that several mutations are responsible for the adaptation of the virus to efficient replication in chick embryos. There is also evidence to suggest that defective interfering particles may also be involved with the attenuation of measles virus (Calain and Roux, 1988).

As is the case with acute measles infection, although to a lesser extent, exposure to measles vaccine is followed by a transient period of immune suppression (Griffin *et al.* 1994), possibly enabling the establishment of a persistent lymphoid tissue infection which may be responsible for the longevity immunity to measles.

3.5. MOLECULAR METHODS FOR THE DETECTION OF MEASLES RNA

In conditions associated with persistent measles virus infection (such as SSPE, otosclerosis and Paget's disease) electron microscopy has initially been used to identify paramyxovirus nucleocapsid-like particles in diseased tissue (Legg, 1967). Subsequently, immunohistochemical techniques have been used to confirm the presence of measles antigens and to study the distribution of measles antigens in diseased tissue (McKenna and Mills, 1989; Legg, 1967). *In situ* hybridisation has been used to determine the presence of measles RNA, to ascertain the location and type of infected cells, and to study the expression of different measles RNA species (Basle *et al.* 1986; McQuaid *et al.* 1993).

Due to the limitations of *in situ* hybridisation, in terms of specificity and sensitivity, RT-PCR for measles RNA has been used to confirm ~~in~~ immunohistochemical findings and provide definitive evidence of measles infection in diseases where an association has been postulated (Kawashima *et al.* 1996b; Reddy *et al.* 1995b; Niedermeyer and Arnold, 1995b).

3.5.1. Target for measles RNA amplification

Measles N-gene RNA is the choice amplification target in IBD tissue for several reasons:

1. Measles virus nucleocapsids have been identified in IBD tissue using electron microscopy (Wakefield *et al.* 1993; Knibbs *et al.* 1993) and measles nucleoprotein has been identified in IBD tissue using immunohistochemistry and immunogold electron microscopy (Wakefield *et al.* 1993; Miyamoto *et al.* 1995; Lewin *et al.* 1995; Ekbom *et al.* 1996; Daszak *et al.* 1997; Wakefield *et al.* 1997). Furthermore, measles N-gene RNA sequences have been detected in a majority of IBD tissues analysed using *in situ* hybridisation (Wakefield *et al.* 1993).
2. A gradient of transcription of measles mRNA occurs in measles-infected cells (Barrett *et al.* 1991) and this gradient has been shown to be exaggerated in persistently infected SSPE tissue (Sidhu *et al.* 1994). Therefore measles N-

gene RNA is the most abundant measles RNA species in infected tissue, and therefore the most likely measles RNA species to be detectable.

3. Several studies have successfully used measles N-gene RNA as a target for measles RNA amplification (Reddy *et al.* 1995; Reddy *et al.* 1996).

In cases of measles virus persistence, mutations may occur which interfere with the efficient amplification of certain viral RNA sequences. For this reason, it is useful to use a second measles RNA sequence, such as measles H-gene RNA, as another target for amplification to provide further evidence as to the presence or absence of measles RNA in IBD tissue. RT-PCR for measles H-gene RNA has been used to determine the presence of persistent measles infection in other conditions (Kawashima *et al.* 1996b).

Choice of material. For RNA viruses such as measles where no viral DNA should be present, amplification methods must be RNA-based. Measles genomic and antigenomic RNA species do not contain the poly-A tails of mRNA (Barrett *et al.* 1991), therefore extraction of total RNA from clinical samples is necessary to aid the efficient detection of all measles RNA species.

3.5.2. RNA-based amplification reactions

RT-nested PCR. In recent years, techniques capable of amplifying nucleic acid sequences have been developed, enabling the sensitive, specific and rapid detection of specific DNA or RNA from clinical samples (Bruce, 1993). PCR using *Taq* DNA polymerase, has enabled the amplification of specific DNA sequences (Saiki *et al.* 1988). In the case of RNA templates, a reverse transcriptase enzyme derived from a retrovirus (such as MMLV or AMV), must first be used to transcribe the RNA target to a complementary DNA (cDNA) strand which can then be amplified using PCR. This reaction uses either specific primers to generate specific cDNA; random primers to generate a random pool of cDNA; or an oligo-dT primer to generate a pool of cDNA corresponding to expressed mRNA. However, the efficiency of RT reactions is low and only a minority of the population of RNA molecules is reverse transcribed to cDNA (Zhang *et al.* 1991). As a consequence, the sensitivity of RT-PCR as a method

can be limited. This therefore ideally requires the quantification of the methods' detection sensitivity prior to use, especially in cases where it is likely that particular RNA species are present at low copy number.

PCR utilises two oligonucleotide primers which bind upstream and downstream to specific DNA target sequences. A thermostable DNA polymerase, such as *Taq* DNA polymerase (from *Thermus aquaticus*), extends these oligonucleotides using dNTPs also present within the reaction mixture until a copy of the original DNA sequence has been synthesised. Repeated thermal cycling (typically 30 cycles) of the reaction mixture permits synthesis of more DNA copies in what is theoretically an exponential manner. When high annealing temperatures are used for thermal cycling, oligonucleotides may bind to DNA in a specific manner. The mechanism of RNA amplification by RT-PCR is shown in Figure 3.4.

In addition, a second nested PCR (nPCR; using an internal primer pair) or semi-nested PCR (using one internal primer and one external primer) can be used to increase sensitivity and specificity of DNA amplification (Mullis and Faloona, 1987).

RT-PCR has been used extensively for the detection of measles RNA in clinical samples of patients with acute measles in order to confirm disease diagnosis and to determine the strain and origin of virus (Shimizu *et al.* 1993; Matsuzono *et al.* 1994; Kawashima *et al.* 1996).

rTth-mediated RT-PCR. Among alternative RT-PCR methodologies has been a combined RT and PCR reaction using *rTth* DNA polymerase - a recombinant enzyme from *Thermus thermophilus* - in a single buffer system (Myers and Sigua, 1997). Using this method, RNA is reverse transcribed by the enzyme in a bicine buffer at an elevated temperature and then thermally cycled to allow nucleic acid amplification. This combined reaction provides several advantages over a conventional two-step RT-PCR reaction:

1. Firstly this method is convenient since only one reaction mixture needs to be prepared for each sample.
2. Secondly, the RT reaction with *rTth* DNA polymerase and EZ buffer is faster than that carried out by either MMLV or AMV reverse transcriptase, enabling

samples to undergo RT-PCR in a relatively short time (under four hours).

3. Thirdly, the elevated temperature used for RT by *rTth* DNA polymerase (60°C) compared with that of MMLV or AMV reverse transcriptase (37-42°C) reduces any transcription problems encountered with RNA secondary structure leading to more efficient RT.
4. Fourthly, a coupled RT-PCR reaction may represent a more efficient method of RNA amplification than a two-step RT-PCR reaction since all the reagents are in the same reaction vessel throughout this method.
5. Finally, the transfer of RT reaction products into PCR reaction mixtures during two-step RT-PCR reactions may lead to contamination with PCR products from previous reactions. Since these PCR products can act as templates for new amplification reactions, contamination leads to false-positive results (Persing, 1991). The probability of PCR contamination is therefore reduced using a combined RT-PCR method.

NASBA. NASBA - an isothermal RNA-based amplification technique - has been applied successfully to the detection of a variety of RNA templates since its development by Guatelli *et al.* in 1990 (van der Vliet *et al.* 1993; van Gemen *et al.* 1993). It has been suggested that NASBA is a more efficient method of amplification than RT-PCR since it is a continuous, isothermal amplification reaction which is not limited by thermal cycling times (Compton, 1991). In addition, NASBA has been shown to be more sensitive than RT-PCR for the detection of HIV RNA in clinical samples (Vandamme *et al.* 1995; Coste *et al.* 1996).

A full description of the NASBA method is provided by Kievits *et al.* (1991); briefly, AMV reverse transcriptase, RNase H and T7 RNA polymerase are used in combination to achieve amplification; AMV reverse transcriptase reverse transcribes the target RNA using a downstream primer linked to a T7 RNA polymerase promoter; RNase H digests the template RNA from the newly formed RNA/DNA hybrid, and AMV reverse transcriptase then uses an upstream primer to synthesise the second DNA strand. The double-stranded DNA product thus formed contains a T7 RNA polymerase promoter site and can be used, therefore, as a template by T7 RNA polymerase to continually

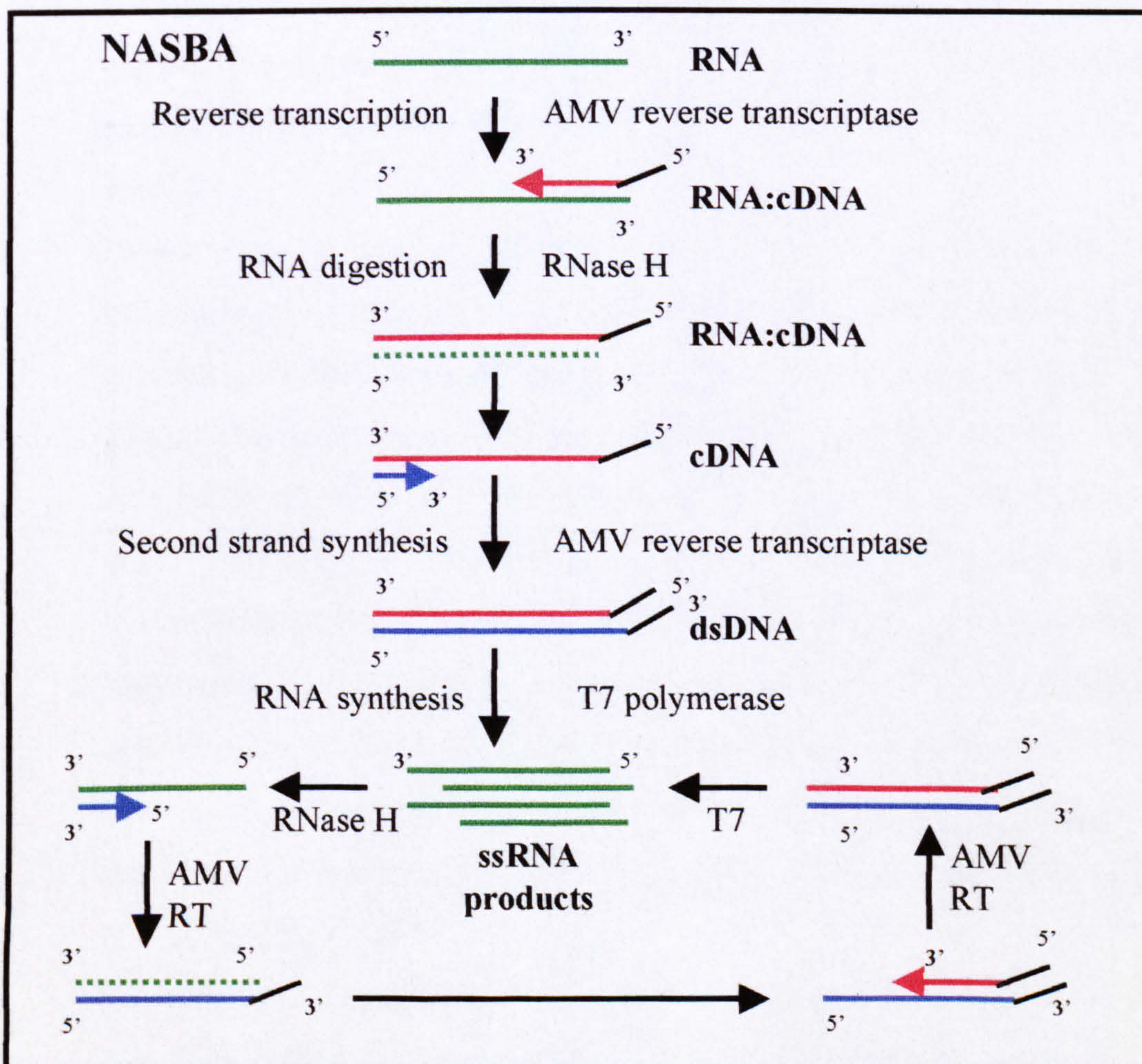
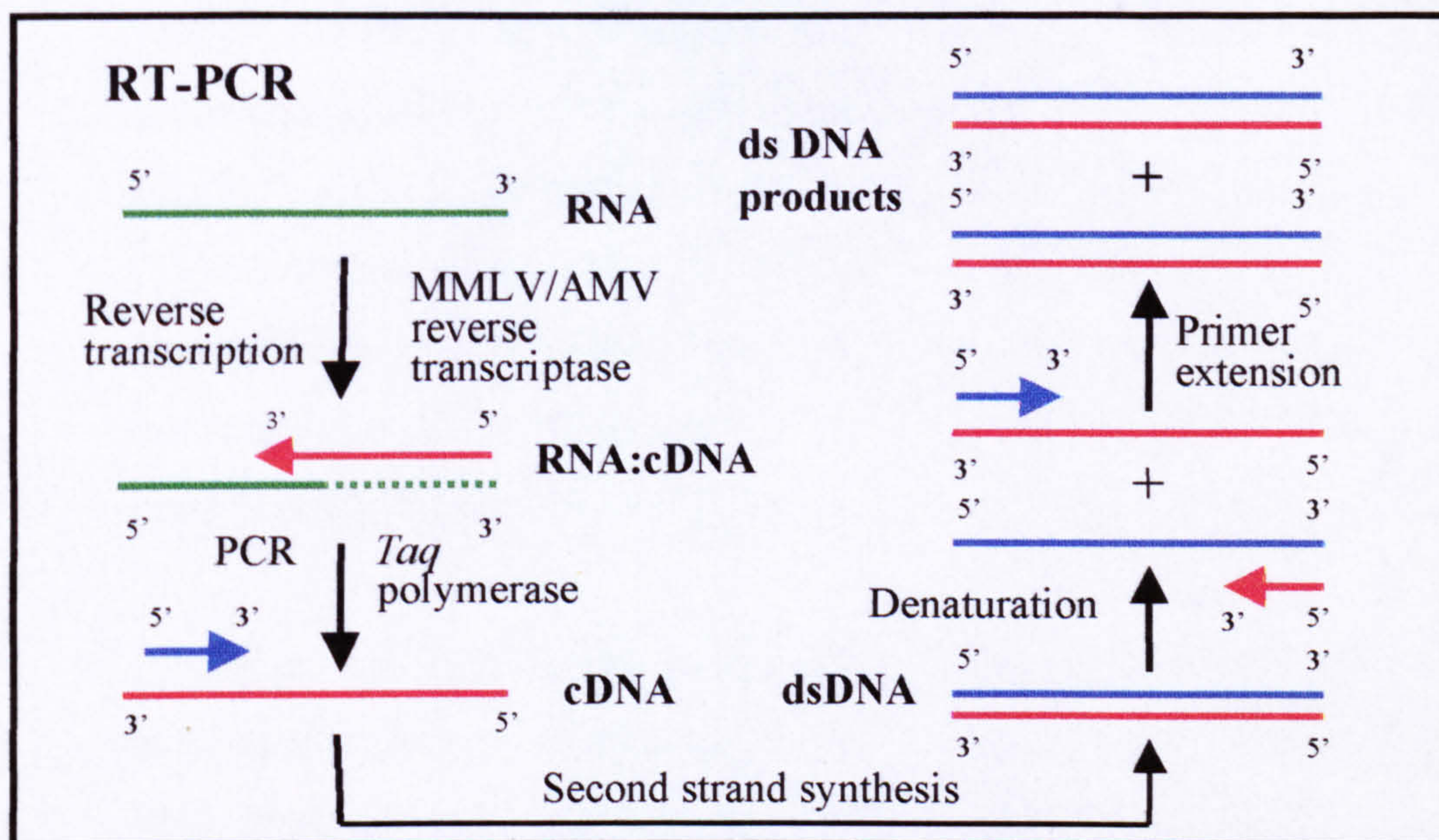
synthesise large numbers of RNA transcripts. Each transcript acts as a new template during the NASBA reaction. Typically 10^{12} to 10^{13} RNA transcripts are synthesised during a NASBA reaction. The mechanism of RNA amplification by NASBA is shown in Figure 3.4.

Due to the isothermal nature of this reaction, non-specific primer annealing and hence non-specific amplification frequently occurs. As a consequence, gel electrophoresis followed by northern blotting and hybridisation with an internal probe, is required to determine the specificity of reaction products.

RNA reaction products may be further amplified using RT-PCR to generate DNA products which can be cloned into plasmid vectors for convenient sequencing and long-term storage. Alternatively, RNA products may be reverse transcribed to cDNA followed by the synthesis of a second DNA strand to enable cloning and sequencing.

Quantification of reaction sensitivity. To assess the ability of a method for measles detection, quantification of measles virus is often performed using a plaque-forming assay, which determines the number of plaque-forming units (PFU) of measles virus in a given homogenate of measles-infected cells (Koschel *et al.* 1995). However, this method cannot accurately quantify the number of measles RNA templates in a sample i.e. it cannot quantify in a molecular genetic fashion the presence of measles virus RNA. An option to address this is to synthesise a measles RNA transcript which can be quantified in terms of numbers of molecules per unit volume and used as a target for amplification. It is also necessary to use total RNA from measles-infected cells, and from measles vaccine samples as templates for amplification in order to confirm the ability of a method to detect a range of measles RNA types.

Figure 3.4: RT-PCR and NASBA



3.5.3 Detection of low copy number measles RNA

Results generated from immunohistochemistry, immunogold, and *in situ* hybridisation experiments indicate the presence of a low copy number, persistent measles virus infection, restricted to the foci of granulomatous inflammation in Crohn's disease (Wakefield *et al.* 1993; Lewin *et al.* 1995; Daszak *et al.* 1997). The failure of several PCR studies to detect measles RNA in IBD tissues implies that if measles RNA is present, extremely sensitive detection methodologies must be employed to permit its detection.

Hybrid capture. Amplification reactions are inhibited by large amounts of RNA or DNA (Pikaart and Villeponteau, 1993), therefore following the isolation of RNA from diseased tissues, sample RNA must be diluted to a working concentration, reducing the measles RNA detection sensitivity. This may explain several groups have failed to detect RNA in IBD tissue using RT-PCR (Smith, 1994; Lewey *et al.* 1995; Iizuka *et al.* 1995; Haga *et al.* 1996; Fan *et al.* 1996).

Hybrid capture, using oligonucleotides (capture sequences) bound to paramagnetic, solid-phase supports, has been used successfully for the detection of a range of nucleic acid species, including measles RNA (Albretsen *et al.* 1990). The detection of low copy number enterovirus RNA from clinical samples of patients with cardiac disease has been aided by hybrid capture (Muir *et al.* 1993) and this method, coupled with RT-PCR has been used to quantify human immunodeficiency virus RNA expression *in vitro* (Volsky *et al.* 1990). Magnetic bead hybrid capture has been shown to improve the sensitivity of Hepatitis C virus RNA detection in blood samples (van Doorn *et al.* 1994), has been used for the isolation of mRNA from clinical samples (Hornes and Korsnes, 1990) and for the purification of phage DNA from bacterial cell lysates (Fry *et al.* 1992).

As a consequence, in order to detect any such low copy-number measles RNA, it may be necessary to enrich total RNA from diseased tissue for measles RNA prior to amplification reactions. Such an enrichment may be performed by hybrid capture using measles-specific oligonucleotides linked to magnetic solid-phase supports. Elutants from these supports may contain purified measles RNA

and can be used for measles RNA amplification.

Virus culture. Alternatively, isolation of the virus from clinical samples, and propagation in African green monkey kidney Vero cells may generate detectable amounts of measles RNA. Culturing of virus from clinical samples may provide a source of replicating virus which can then be used to study the functional characteristics of any virus recovered. However, as in the case of SSPE, a defective form of measles virus may be present which cannot be cultured.

3.5.4. Mumps and rubella

Mumps is an acute infection that occurs primarily in children, characterised by swelling of salivary glands. Meningitis is an important complication, occurring in up to 10% of acute cases (Baum and Litman, 1996). Vaccination, using live attenuated virus, was introduced in 1967 and is now incorporated into the MMR triple vaccine. Mumps virus belongs to the paramyxovirus genus and ^{has} a similar structure and replicative cycle to that of measles virus (Galinski and Wechler, 1991).

Rubella is an RNA virus belonging to the *togaviridae* family (Gershon, 1996). Rubella is an acute infection of children and adults, characterised by fever and rash. Congenital rubella can result in a range of transient, permanent and developmental manifestations including mental retardation. Vaccination, using a live, attenuated virus was introduced in 1969 and is now incorporated into the MMR triple vaccine.

Both mumps and rubella viruses are non-retroviral RNA viruses, therefore, as is the case with measles virus, molecular detection methods are RNA-based.

3.6. HYPOTHESIS

Observations of the behaviour of measles virus have led to the hypothesis that, as is the case with SSPE (Miller *et al.* 1992), early exposure to measles virus in some individuals (possibly with a host immune dysfunction) leads to viral persistence and susceptibility to IBD later in life (Pounder, 1994; Pounder, 1995; Wakefield *et al.* 1995). This hypothesis proposes that early exposure to measles virus (wild-type or vaccine) leads to a persistent infection of intestinal follicular dendritic cells in some individuals. An environmental or genetic event later in life triggers virus replication and infection of lymphocytes which come into contact with infected dendritic cells. Infected circulating lymphocytes home to the intestine via the submucosal vasculature, infecting endothelial cells during infiltration.

Infected submucosal endothelial cells express measles antigens triggering aggregation of circulating mononuclear cells, including monocytes, macrophages and lymphocytes, and leading to occlusion of submucosal microvasculature, ischaemia and infarction. Tissue damage is then followed by invasion of opportunistic luminal bacteria, leading to chronic inflammation. Ongoing vasculitis, ischaemia and secondary bacterial infection leads to the perpetuation of this chronic inflammation.

Infection of follicular dendritic cells by measles virus or a secondary pathogen may also be involved with the perpetuation of chronic inflammation via inappropriate IL-12 production and a disruption of gut homeostasis.

3.7. AIMS OF PROJECT

1. A comparison of the relative sensitivity and fidelity of molecular amplification techniques, and the development of new methodologies for measles RNA detection will be performed to determine the optimum method for the identification of any measles RNA in IBD clinical samples.
2. This optimised method will be used to determine the presence of measles RNA/PBMCs and resection tissue from patients with IBD and controls. Sequence analysis will be used to characterise any measles-specific amplification products from the samples analysed to determine the strain and origin of virus detected.
3. Clinical samples will be analysed from patients with autistic enteropathy for the presence of measles, mumps and rubella RNA to investigate an association between MMR vaccination and the development of this syndrome.

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Chapter 4

Chapter 4. MATERIALS AND METHODS

4.1. MATERIALS

4.1.1. Chemicals, biologicals and enzymes

Unless otherwise stated, all chemicals, of both analytical and reagent grade, and biologicals were obtained from the Sigma Chemical Co. (Poole, UK) or BDH Ltd. (Dagenham, UK). Restriction enzymes were obtained from New England Biolabs. (Hitchin, UK) and radiolabelled nucleotides and "ThermoSequenase" cycle sequencing kits from Amersham International (Amersham, UK). dNTPs for PCR reactions and "Total RNA isolation reagent" were obtained from Advanced Biotechnologies (Leatherhead, UK). *rTth* DNA polymerase and "EZ buffer" were obtained from Perkin Elmer (Warrington, UK). MMLV reverse transcriptase was obtained from Gibco BRL (Paisly, UK) as was T4 DNA ligase, *Taq* DNA polymerase, random hexamers and acrylamide gel mix solution. Alkaline phosphatase and DNaseI were obtained from Boehringer Mannheim (Lewes, UK) and NASBA reagents from Organon Technika (Boxtel, The Netherlands). T4 DNA polymerase, ribonuclease inhibitor, T7 transcription kits and T4 polynucleotide kinase were obtained from Helena Biosystems (Sunderland, UK) and oligonucleotides for PCR, NASBA and hybrid capture were supplied either by Perkin Elmer, Organon Technika or the University of Greenwich (London, UK). "Lymphoprep" solution was obtained from Nycomed (Oslo, Norway). Finally, "Copy kit" cDNA synthesis kits were obtained from Invitrogen (Leek, The Netherlands) and "Wizard" PCR and plasmid purification kits were obtained from Promega (Southampton, UK).

Table 4.1: Bacteria, viruses and cells

Bacteria/virus/ cell	Strain/Genotype	Source
MAX efficiency <i>E.coli</i> cells	F ⁻ ϕ 80dlacZ Δ M15 Δ (lacZYA- argF)U169 <i>deoR recA1endA1</i> <i>hsdR17</i> (r _K ⁻ ,m _K ⁺) <i>phoA supE44</i> λ ⁻ <i>thi-1 gyrA96 relA1</i>	Gibco BRL (Paisley, UK)
African green monkey kidney Vero cells	Catalogue number 84113001; susceptible to a range of viruses, including measles mumps, and rubella	European Collection of Cell Cultures (Porton Down, UK)
Measles virus	Hu2 strain, from a clinical isolate	Gift from B.K. Rima, Queen's University Belfast, Northern Ireland
Measles vaccine	Schwarz strain; >1,000 plaque forming units of virus per dose, supplied as a lyophilised solid derived from infected chick embryo fibroblasts	Evans Medical (Horsham, UK)

Table 4.2: RNA and DNA

RNA/DNA	Description	Source
Mumps RNA	Total RNA isolated from the supernatant of Vero cells infected with the VR 106 strain of mumps virus	Gift from Professor Enders (Stuttgart, Germany)
Rubella RNA	Total RNA isolated from Vero cells infected with the M33 strain of rubella virus	Gift from Professor Enders (Stuttgart, Germany)
pT7BlueE	T/A cloning vector	AMS Biotechnology (Witney, UK)
HIV1 gag1 DNA	144 bp <i>PstI</i> / <i>SphI</i> restriction endonuclease fragment	Gift from B. van Gemen (Organon Technika)

Table 4.3: Sizes of RNA and DNA size markers in base pairs

G3151 RNA marker (Promega)	0.24-9.5 kb RNA marker (Gibco BRL)	ϕ X174/ <i>HaeIII</i> DNA marker (Gibco BRL)	1 kb DNA marker (Gibco BRL)	100 bp DNA marker (Helena Biosystems)
9488	9490	1353	12216 1636	1000
6225	7460	1078	11198 1018	900
3911	440	872	10180 517	800
2800	2370	603	9162 516	700
1898	1350	310	8144 396	600
872	240	281	7126 344	500
562		271	6108 298	400
363		234	5090 220	300
		194	4072 201	200
		118	3054 154	80
		72	2036 134	
			75	

4.1.2. Buffers and solutions

All buffers and solutions were made up in 1 litre of double deionised water (dd H₂O), unless otherwise indicated, and sterilised by autoclaving at 120°C for 30 mins.

Table 4.4: Buffers and solutions

Buffer/solution	Description
Bromophenol blue/xylene cyanol solution	1 ml of solution was mixed with 4 ml of formamide before use as a 6x loading dye.
Denaturing solution	0.5 M sodium hydroxide and 1.5 M sodium chloride
Fetal Bovine Serum (FBS)	Heat inactivated; sterile
Fix solution	5% (v/v) acetic acid and 15% (v/v) methanol. Not autoclaved
Hybridisation buffer	5x SSC (v/v), 0.2% polyvinylpyrrolidone (w/v), 0.2% (w/v) BSA, 0.2% (w/v) ficoll, 8 mM Na ₂ HPO ₄ , 13 mM NaH ₂ PO ₄ and 7% (w/v) SDS. Not autoclaved
(MOPS)-EDTA-sodium acetate buffer	10x stock contains 0.4 M MOPS pH, 7.0, 0.1 M sodium acetate, 10 mM EDTA
Neutralisation solution	1 M tris and 1.5 M sodium chloride in ddH ₂ O, adjust pH to 7.4 with concentrated HCl
Phosphate buffered saline (PBS)	8 g sodium chloride, 0.2 g potassium chloride, 1.44 g Na ₂ HPO ₄ , 0.24 KH ₂ PO ₄ , Adjust pH to 7.4 with concentrated HCl
RNA loading buffer	1.5 ml of stock loading buffer contains 750 µl deionised formamide, 240 µl 37% (v/v) formaldehyde, 150 µl 10x MOPS-EDTA-sodium acetate buffer, 180 µl DEPC-treated ddH ₂ O, 100 µl glycerin and 80 µl 10% (w/v) bromophenol blue. Not autoclaved
Saline sodium citrate (SSC)	20x stock; 175.3 g sodium chloride, 88.2 g sodium citrate per liter of ddH ₂ O. Adjust the pH to 7.2 with concentrated NaOH
Tris-acetate-EDTA (TAE)	50x stock; 242 g tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0)
Tris-borate-EDTA (TBE)	5x stock; 54 g tris, 27.5 g boric acid, 20 ml 0.5 M EDTA, pH 8.0
Trypan blue	0.4 (w/v) % sterile solution.
Wash buffer	3x (v/v) SSC and 1% (w/v) SDS. Not autoclaved
Washing/binding (WB) buffer	1 M sodium chloride, 10 mM tris and 1 mM EDTA in DEPC-treated ddH ₂ O. Adjust the pH to 7.4 using concentrated HCl

Table 4.5: Culture media

Culture media	Description
RPMI 1640 media	Sterile, filtered, endotoxin tested, without glutamine. 5 ml 200 mM L-glutamine. 50,000 U penicillin and 50 mg streptomycin were added per liter before use
Dulbecco's modified Eagles media (Gibco BRL; Paisley, UK)	Sterile, filtered, with 4.5 g l ⁻¹ glucose, without sodium pyruvate. 50,000 U penicillin, 50 mg streptomycin and 100 ml FBS were added per liter before use
Lymphoprep solution	Obtained sterile and stored in the dark
Luria-Bertani (LB) media	10 g bacto-tryptone, 10 g sodium chloride, 5 g bacto-yeast extract in 1 liter ddH ₂ O. Sterilised by autoclaving
Agar plates	15 g bacto-agar per liter of sterile LB media and autoclaved. The solution was cooled to 50°C before the addition of ampicillin to a final concentration of 50 µgml ⁻¹ and then poured into sterile agar plates (30 ml per plate) and left to set. For blue/white colony screening, 35 µl of 50 mgml ⁻¹ Xgal and 20 µl of 100 mM IPTG were spread over the surface of plates and allow to dry before use
SOC media	10 g bacto-tryptone, 5 g bacto-yeast extract, 0.5 g sodium chloride and 10 ml of 250 mM potassium chloride in 1 liter dH ₂ O, adjusted pH to 7.0 with concentrated NaOH and sterilise by autoclaving. 20 ml of sterile 1 M glucose solution and 5 ml sterile 2 M magnesium chloride were added before use.

Table 4.6: Equipment and consumables

Equipment	Supplier	Address
Cell culture flasks	Fisons	Loughborough, UK
Eppendorf tubes	Fisons	Loughborough, UK
Filter tips	Advanced Biotechnologies	Leatherhead, UK
Haemocytometer	Fisons	Loughborough, UK
Horizon electrophoresis tanks and accessories (models 11:14 and 58)	Gibco	Paisley, UK
Magnetic stands	Dynal	Wirral, UK
Micropestels (sterile, disposable)	BDH Ltd.	Dagenham, UK
Mini-vertical electrophoresis system	Biorad	Hemel Hempstead, UK
Nylon membranes (Hybond-N)	Amersham International	Amersham, UK
PCH-2 thermal cycler	Techne	Cambridge, UK
Polaroid film (black and white; type 667)	GRI	Dunmow, UK
Sequencing gel unit and accessories	Amersham International	Amersham, UK
Ultra Turax T25 rotor and 8 mm dispersing shaft	Marathon Laboratory Supplies	London, UK
Ultraviolet (UV) transilluminator	UVP	San Gabrielle, USA
Uvikon spectrophotometer	Kontron Instruments	Watford, UK
X-omat automated x-ray processor	Kodak	San Fransisco, USA
X ray film (Fuji RX; 20x30 cm and 30x45 cm)	GRI	Dunmow, UK

4.2. METHODS

4.2.1. General considerations

RNA is a highly labile molecule and susceptible to degradation by ribonucleases. Ribonucleases are ubiquitous in living cells and highly resistant to heat inactivation. For this reason, all equipment and most reagents used in experiments involving RNA were treated in such a way as to destroy any ribonucleases present.

DEPC was used to deactivate ribonucleases which it does by virtue of its ability to modify histine and tyrosine residues in proteins. DEPC was added to double deionised water (ddH₂O) to a concentration of 1% (v/v) and the solution left to mix with a magnetic bar for 16 hrs at 20°C. The mixture was then autoclaved to remove DEPC. All glassware was cleaned with detergent, washed with tap water followed by ddH₂O, baked at 180°C overnight and wrapped in aluminium foil prior to use. Prior to use, electrophoresis tanks were filled with 3% hydrogen peroxide for 2 hrs, then rinsed with DEPC-treated ddH₂O. Plasticware was soaked overnight in water containing 1% (v/v) DEPC then autoclaved, wrapped in aluminium foil and dried and prior to use. Disposable nuclease-free pipette filter tips were used in all experiments involving RNA.

All RNA isolation procedures were performed in a 4°C cold room in order to protect RNA from potentially damaging enzyme activity.

All reagents and buffers for DNA work were made up in ddH₂O and autoclaved whenever possible. Sterile Eppendorf tubes were used.

4.2.2. Cell culture and virus propagation

Cell culture and PBMC isolation experiments, and measles virus propagation experiments were performed in separate laboratories in order to minimise the possibility of contamination. All cell culture experiments were performed in sterile hoods and incubations performed in 5% CO₂ at 37°C.

In order to permit the identification of any measles, mumps or rubella in PBMC samples, PBMCs were cocultivated with donor PBMCs and/or African green monkey kidney Vero cells. Cocultivation aids the isolation of virus by increasing the amount of virus present.

Cultivation of PBMCs. PBMCs were isolated from blood samples as described in section 4.2.3. Pellets of PBMCs were resuspended in 5 ml of RPMI 1640 media and 50 μ l of this suspension mixed with 50 μ l of tryptophan blue solution. 50 μ l of this mixture was placed on a haemocytometer slide and live cells counted by phase contrast microscopy. 10^6 PBMCs were used for cultivation experiments. The remaining PBMC suspension was centrifuged at 400 g for 10 mins and PBMCs resuspended in 1 ml of 10% DMSO in FBS and stored at -70°C until required.

10^6 PBMCs were made up to a volume of 5 ml with RPMI 1640, transferred to a sterile plastic flat-bottomed 25 cm^2 cell culture flask and phytohaemagglutinin (PHA)-L was added to a final concentration of $5\text{ }\mu\text{gml}^{-1}$. After three days of incubation, cells were transferred to a sterile 20 ml universal tube and centrifuged at 400 g for 30 mins. The resulting pellet was resuspended in 4 ml of RPMI 1640 media and split into four equal aliquots:

1. One aliquot was resuspended in RPMI 1640 media containing 2 Uml^{-1} IL-2 and cells passaged three times to stimulate viral replication. Media was changed three times per week and after each passage cell stocks were stored at -70°C for total RNA preparation.
2. One aliquot was stored in 10% FBS in DMSO at -70°C as described above.
3. One aliquot was cocultivated with donor PBMCs to stimulate viral replication. Live cells were counted as described above and 10^5 PHA-L-stimulated PBMCs were seeded with 10^5 PBMCs from a healthy donor in one well of a sterile 24 well plate. Cells were incubated for three days in 2 ml of RPMI 1640 media containing 2 Uml^{-1} IL-2. Cells were then centrifuged and either used directly for total RNA preparation or cocultivated with Vero cells.
4. One aliquot of PBMCs was cocultivated with Vero cells as described below.

Cultivation of Vero cells. Vero cell stocks, containing 10^6 cells were thawed at 37°C , added to 10 ml of Dulbecco's modified Eagle's media and centrifuged at 400 g for 10 mins. Cell pellets were then resuspended in 20 ml Dulbecco's modified Eagle's media, seeded in sterile plastic flat-bottomed 75 cm^2 cell culture flasks and media changed every three days. At confluence cells were

detached from the flask by the addition of 5 ml of trypsin/EDTA solution and incubated for 10 mins at 37°C. 10 ml of Dulbecco's modified Eagle's media^{um} was then added and the solution transferred to a sterile 50 ml Falcon tube. The suspension was centrifuged, cell pellets were resuspended in 10 ml of Dulbecco's modified Eagle's media^{um}, and live cells were counted as described above.

Cocultivation of Vero cells and PBMCs. 10⁵ Vero cells, in 5 ml of Dulbecco's modified Eagle's media^{um}, were added to one well of a sterile, plastic 24 well plate and incubated. At subconfluence, media^{um} was replaced by either PBMCs or PBMC cocultures/mixed 1:1 with Dulbecco's modified Eagle's media.

After a 1 hr incubation at 37°C, Vero cell coculture supernatants were discarded, Vero cells washed with PBS, and 5 ml of fresh Dulbecco's modified Eagle's media^{um} added. Media^{um} was changed three times per week and cells passaged up to seven times by trypsinisation, centrifugation and resuspension of cells as described above. Cells were checked for cytopathic effects (characteristic of measles virus infection) at each passage and aliquots of cells were stored at -70°C after each passage. Cell^s derived from individual PBMC samples were pooled, centrifuged and pellets used directly for total RNA preparation as described in section 4.2.3.

Propagation of measles virus. Measles-infected Vero cells were used as a source of measles RNA for use as a positive control in measles RNA amplification reactions. Sterile 75 cm² flasks containing confluent monolayers of Vero cells were obtained as described above. Measles virus stocks (lysates of previously infected cell cultures) were diluted 1:10. Dulbecco's modified Eagle's media^{um} was replaced by 2 ml of diluted virus solution and Vero cells and virus left to incubate for 2 hrs at 37°C. 8 ml of Dulbecco's modified Eagle's media^{um} was then added and the infected cells incubated for between 1 and 3 days until a clear cytopathic effect was observed. Cells were then scraped into sterile 20 ml universal tubes, centrifuged at 400 g and the resulting pellets used directly for total RNA preparation as described in section 4.2.2. Aliquots of infected cell pellets were also resuspended in 1 ml Vero media^{um} and stored at -70°C for future

measles virus infections.

4.2.3. RNA purification

Cells. Cultured Vero cell monolayers were scraped from flasks, transferred to a 50 ml sterile Falcon tube and centrifuged at 600 g for 10 mins at 20°C. The supernatant was aspirated and cell pellet resuspended in 3 ml of "Total RNA isolation reagent" by gentle pipetting, and the equal volumes of the emulsion transferred to two 1.5 ml sterile Eppendorf tubes. Total RNA was isolated according to the manufacturer's instructions (based on a method developed by Chomczynski and Sacchi, 1987). Total RNA pellets were resuspended in 50 µl of DEPC-treated ddH₂O and RNA concentration determined spectrophotometrically (section 4.2.8.). 30 U of ribonuclease inhibitor was added and samples stored at -70°C until required.

Measles Vaccine. Four doses of a lyophilised sample of measles vaccine were resuspended in 1 ml of "Total RNA isolation reagent" and RNA isolated according to the manufacturer's instructions. RNA concentration and purity was determined spectrophotometrically (section 4.2.8.).

Blood. 10 ml of blood was taken from IBD, autistic enteropathy and control patients in EDTA-coated tubes. Blood samples were transferred to sterile 20 ml universal tubes and mixed with equal volumes of sterile PBS. Blood/PBS mixtures were gently laid onto 0.5 volumes of sterile lymphoprep solution (in sterile 20 ml universal tubes) using a sterile Pasteur pipette and centrifuged at 600 g for 30 mins at 20°C. Buffy coats containing PBMCs were aspirated using sterile Pasteur pipettes and transferred to fresh sterile 20 ml universal tubes which were filled with PBS. The suspensions were centrifuged at 600 g for 30 mins at 20°C. Supernatants were discarded and the PBMC pellets gently resuspended in 1 ml of "Total RNA isolation reagent". Total RNA was isolated according to the manufacturer's instructions. Isolated RNA pellets were resuspended in 30 µl DEPC-treated ddH₂O and RNA concentration and purity determined spectrophotometrically.

Resection tissue. Intestinal resection tissue samples were obtained from IBD or control patients undergoing surgical removal of diseased sections of large or small bowel. Areas of intestinal resection tissue were characterized as macroscopically inflamed or normal and full-thickness intestinal resection tissue was divided into approximately 500 mg segments. Tissue samples were placed in sterile 2 ml cryovials and snap-frozen on liquid nitrogen prior to storage at -70°C. In all cases, the post-resection time until snap freezing was less than 5 mins.

Frozen resection tissue samples were transferred to a sterile 50 ml Falcon tube containing 3 ml of "Total RNA isolation reagent" and homogenised using an Ultra Turrax rotor and 8 mm dispersing shaft at 15,000 rpm for 2 mins. The emulsion was transferred to two sterile 1.5 ml Eppendorf tubes, total RNA was isolated according to the manufacturer's instructions, and RNA concentration and purity determined spectrophotometrically (section 4.2.8.).

Biopsy samples. Intestinal pinch biopsy samples were obtained from autistic enteropathy patients undergoing colonoscopy. Biopsies were transferred to sterile 2 ml cryovials, placed in liquid nitrogen then stored at -70°C within 5 mins of biopsy removal.

Frozen biopsies were transferred to sterile 1.5 ml Eppendorf tubes containing 1 ml of "Total RNA isolation reagent" and a micropestel used to manually grind the biopsy into an emulsion. Total RNA was isolated according to the manufacturer's instructions and RNA concentration and purity determined spectrophotometrically (section 4.2.8.).

In vitro RNA transcripts. *In vitro* transcription reactions were deproteinised as follows: an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the RNA transcript-containing solution in a sterile 1.5 ml Eppendorf tube and the mixture vortexed and centrifuged at 12,000 g for 5 mins at 20°C. The upper aqueous phase (containing RNA transcripts) was transferred to a fresh sterile 1.5 ml Eppendorf tube and 1 volume of cold isopropanol added. RNA was left to precipitate at -70°C for 16 hrs then centrifuged at 12,000 g for 15 mins at room temperature. The supernatant was discarded and the RNA pellet

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washed twice with 70% (v/v) ethanol, air dried and resuspended^{ed} in 50 µl of DEPC-treated ddH₂O. Total RNA concentration and purity was determined spectrophotometrically (section 4.2.8.).

The molecular mass of RNA transcripts was determined from their length and from this, concentrations of RNA transcripts were calculated in terms of molecules µl⁻¹. 30 U of ribonuclease inhibitor were added and transcript RNA serially diluted in DEPC-treated ddH₂O and stored at -70°C until required. These dilution series were subsequently used in later experiments to quantify the sensitivities of measles RNA detection methods (Chapters 5 and 8).

4.2.4. Hybrid capture for measles N-gene RNA

Magnetic solid-phase supports (MSPSs) were prepared according to a previously described method (Davies *et al.* 1997). 5'-amino modified capture sequences (HC1 and HC2; Table 4.8) were covalently attached to carboxymethyl (CM) -modified MSPS by a water soluble carbodiimide-mediated coupling reaction described below.

CM-MSPS (100 µl settled volume) was washed with 0.2 M morpholineethanesulphuric acid (MES), pH 6 (3 x 200 µl), then resuspended in 200 µl 0.2 M MES/0.1 M 1-(3-dimethylaminopropyl)-3-ethyl carbomide (EDC), pH 6. 0.25 µmol each of the HC1 and HC2 capture sequences (2.5 µl of a 100 mM stock) were added, and the mixture shaken gently at 20°C for 24 hrs. The supports were then washed three times with 0.1 M potassium phosphate buffer (PB; 1 ml each), pH 7, and stored in a further 500 µl PB, pH 7, at 4°C until needed.

50 µl of MSPS-capture sequence suspensions were placed in a sterile 0.5 ml Eppendorf tube. The MSPS was then immobilised using a magnetic stand and the supernatants removed. 50 µl of sample (containing up to 40 µg of total RNA) was diluted with an equal volume of WB buffer (see Materials), heated at 65°C for 2 mins, added to the MSPS and the mixture was shaken gently at 20°C for 5 mins. MSPS was magnetically immobilised, the supernatants removed and MSPS was washed twice with WB buffer (150 µl each). To elute captured RNA, 10 µl of 2 mM EDTA, pH 7.4 was added to the MSPS and the mixture heated at 65°C for 5 mins, the MSPS magnetically immobilised and the RNA-

containing supernatant removed to a fresh sterile 1.5 ml Eppendorf tube and stored at -70°C until required.

4.2.5. RT-PCR

Reverse transcription. Reverse transcription reactions were carried out in 1x RT reaction buffer containing 10 mM DTT, 0.5 mM each dNTP, 2.5 µM random hexamers (or 0.75 µM specific primer - see Table 4.8.), 2 U ribonuclease inhibitor and 200 U MMLV Superscript II reverse transcriptase. The reaction mixture was made up as a "master mix" and dispensed into sterile 0.5 ml Eppendorf tubes in 10 µl aliquots as required.

10 µl of sample RNA (containing up to 5 µg of RNA) was denatured by heating to 70°C for 10 mins, cooled on ice and added to the 10 µl of RT reaction mixture. The mixture was incubated at 37°C for 2.5 hrs followed by a 5 minute incubation at 95°C, to inactivate the MMLV reverse transcriptase. Product cDNAs were stored at -20°C until required for PCR.

PCR. PCR reactions were carried out in 1x PCR reaction buffer containing 1.5 mM MgCl₂, 0.2 mM each dNTP, 250 nM each primer and 1.25 U *Taq* DNA polymerase. Primer details are given in Table 4.8. The reaction mixture was made up as a "master mix" and dispensed into sterile 0.5 ml Eppendorf tubes in 48 µl aliquots as required. Reaction mixtures were overlayed with 50 µl of mineral oil and 2 µl of reverse transcription mixture (see above) was added to each reaction mix. Reactions were cycled on a PCH-2 thermal cycler using the parameters given in Table 4.7.

For nested or semi-nested PCR reactions, second round PCR reaction mixes were made up containing a pair of primers which annealed internally to those used in the first round reactions, and the same reagent final concentrations with 1 µl of first round product in a total volume of 25 µl. The mixtures were overlayed with 40 µl of mineral oil and thermally cycled using the parameters given in Table 4.7.

4.2.6. *rTth*-mediated RT-PCR

rTth RT-PCR reactions were carried out in 1x EZ buffer containing 2.5 mM

Mn(OAc)₂, 300 µM each dNTP, 450 nM each primer and 5 U *rTth* DNA polymerase. Primer details are given in Table 4.8. The reaction mixture was made up as a "master mix" and dispensed into sterile 0.5 ml Eppendorf tubes in 40 µl aliquots as required. Reaction mixtures were overlaid with 50 µl of mineral oil before the addition of 10 µl of sample RNA thermal cycling using the parameters given in Table 4.7.

Table 4.7: PCR cycling parameters.

	Reaction			
	PCR			RT-PCR
Template	Measles N- and H-genes	U1A and mumps P-gene	Rubella E1 gene	U1A, measles N- and H-genes
Step 1	95°C, 5 mins	95°C, 5 mins	95°C, 3 mins	60°C, 30 mins
Step 2	56°C, 30 secs	56°C, 30 secs	60°C, 1 min	95°C, 1 min
Step 3	72°C, 30 secs	72°C, 30 secs	72°C, 1 min	56°C, 1 min
Step 4	95°C, 30 secs	95°C, 30 secs	95°C, 30 secs	60°C, 7 mins
Step 5	72°C, 5 mins	72°C, 5 mins	72°C, 5 mins	
Comments	Steps 2,3 and 4 repeated 35 times followed by 25 times for nested PCR	Steps 2, 3 and 4 repeated 40 times	Steps 2,3 and 4 repeated 40 times followed by 25 times for nested PCR	Steps 2 and 3 repeated 40 times

4.2.7. NASBA

NASBA reaction mixtures were carried out in 1x NASBA buffer containing 250 µM each dNTP, 500 µM each of ATP, UTP and CTP, 375 µM GTP, 125 µM ITP, and 250 µM each primer in DEPC-treated ddH₂O. Primer details are given in Table 4.8. The reaction mixture was made up as a "master mix" and dispensed into sterile 1.5 ml Eppendorf tubes in 10 µl aliquots as required. 5 µl of sample RNA (containing 100 ng total RNA) was added and denatured by heating to 65°C for 5 mins. Reaction mixtures were subsequently transferred to a 41°C water bath for 5 mins and 5 µl of NASBA enzyme mix (which had been previously warmed to 20°C) was added to give final concentrations of 0.1 U RNase H, 32 U T7 polymerase and 6.5 U AMV reverse transcriptase. Reactions were mixed by gentle pipetting and incubated at 41°C for 90 mins.

Table 4.8: Oligonucleotide primer and probe sequences with target/annealing positions.

Oligo	Target	Sequence
Measles N-gene hybrid capture		
HC1	1328-1368	AGA AAT GAT ACT TGG GCT TGT CTG GGT CCA ACC GCT CAT C
HC2	1288-1322	GTT TCA GAG ATT GCA ATG CAAC TAC TGA GGA CAA GAT CAG
Measles N-gene amplification		
MV1	1198-1219	TTA GGG CAA GAG ATG GTA AGG
MV2	1610-1631	GTT CTT CCG AGA TTC CTG CCA
MV3	1248-1269	AGC ATC TGA ACT CGG TAT CAC
MV4	1480-1501	AGC TCT CGC ATC ACT TGC TCT
MV5	1348-1368	AGA AAT GAT ACT TGG GCT TGT
AB20N (NASBA)	1200-1219	AGG GCA AGA GAT GGT AAG GA
AB22N (NASBA)	1358-1379	<i>AAT TCT AAT ACG ACT CAC TAT AGG G</i> GA TCA CCG TGT AGA AAT GAC A
AB10 (HRP)	1288-1308	GTT TCA GAG ATT GCA ATG CA
Measles H-gene RNA amplification		
MF1	7209-7233	GCT TCC CTC TGG CCG AAC AAT ATC G
H3	8106-8125	CAG TCA GTA ATG ATC TCA GC
H6	8677-8701	CTT GAA TCT CGG TAT CCA CTC CAA T
H7	8147-8171	GAG CTC AAA CTC GCA GCC CTT TGT C
H4	8458-8482	ATC CTT CAA TGG TGC CCA CTC GGG A
H5	8370-8392	TCC CGA CAA CAC GAA CAG ATG AC
U1A RNA amplification		
U1A1	583-603	GGC CCG GCA TGT GGT GCA TAA
U1A2	378-400	CAG TAT GCC AAG ACC GAC TCA GA
U1A1N (NASBA)	583-603	<i>AAT TCT AAT ACG ACT CAC TAT AGG GAG A</i> GGC CCG GCA TGT GGT GCA TAA
U1A3 (HRP)	451-472	AGA AGA GGA AGC CCA AGA GCC A
Mumps P-gene RNA amplification		
MUP1	2120-2139	CTC ATT GGC AAT CCA GAG CA
MUP2	2324-2343	ATG AAC CTG TTG GTT GGA TA
MUP3	2181-2200	AAG TCA AAG GGC AGA GGC TC
Rubella E1-gene RNA amplification		
R2	8807-8826	CAA CAC GCC GCA CGG ACA AC
R7	8972-8991	CCA CAA GCC GCG AGC AGT CA
R11	8826-8845	CTC GAG GTC CAG GTC CTG CC
R8C	8949-8968	GAA TGG CGT TGG CAA ACC GG
Sequencing primers (for sequencing of DNA in vector pT7Blue)		
FP	23-43	CTAATACGACTCACTATAGGG
RP	144-162	GTTTCCAGTCACGACGT

4.2.8. RNA and DNA analysis

Agarose gel electrophoresis of RNA. 1.3 g of agarose was added to 100 ml MOPS-EDTA sodium acetate buffer made up in DEPC-treated ddH₂O. This mixture was microwaved until the agarose melted then cooled to approximately 50°C before the addition, in a fume hood, of 5.1 ml 37% (v/v) aqueous formaldehyde. The solution was mixed and poured into either a Horizon 11:14 or a Horizon 58 electrophoresis tank. After setting (approximately 30 mins), the gel was soaked in MOPS-EDTA-sodium acetate buffer for a further 30 mins prior to electrophoresis.

RNA (up to 2 µg in a total volume of 5 µl), 5 µl of NASBA reaction products or 1 µg of an RNA size marker, was added to 10 µl of RNA loading buffer and 1 µl of 1 mgml⁻¹ ethidium bromide and the mixture heated to 70°C for 10 mins to denature the RNA. Samples were immediately loaded into gel wells and electrophoresed at 100 volts for 1 hr. The gel was placed on a ultraviolet (UV) transilluminator and photographed using a Polaroid camera fitted with black and white type 677 film.

Agarose gel electrophoresism of DNA. 1.3 g agarose was added to 100 ml TAE buffer, made up in ddH₂O, and microwaved until the agarose melted. The solution was cooled to approximately 50°C before the addition of 10 µl of 10 mgml⁻¹ ethidium bromide. The solution was mixed and poured into either a Horizon 11:14 or a Horizon 58 electrophoresis tank and left to set for approximately 30 mins.

10 µl of PCR reaction products, or 1 µg plasmid DNA, were mixed with 2 µl bromophenol blue-cyanol solution and loaded into gel wells, and DNA electrophoresis at 100 volts for 1 hr. The gel was placed on a UV transilluminator and photographed as above.

Spectrophotometry. 2 µl of purified DNA or RNA was made up to 300 µl with dd H₂O. This solution was added to a clean 300 µl spectrophotometry cell and the optical density (OD) of the solution was measured at 260 and 280 nm wavelengths using a Uvkon spectrophotometer. Nucleic acid concentration was determined by the OD₂₆₀ value and nucleic acid purity determined by the OD₂₆₀:

OD₂₈₀ ratio.

4.2.9. Nucleic acid hybridisation

Blotting. Following electrophoresis, NASBA reaction products were transferred directly to a "Hybond-N" nylon membrane as described by Sambrook *et al.* (1989a) and RNA was subsequently cross-linked to membranes by 5 mins irradiation on a UV transilluminator. Membranes were stored at 4°C until required.

PCR products were denatured by soaking in 200 ml of denaturing solution for 40 mins at 20°C. The gel was then rinsed with ddH₂O and soaked twice in 200 ml of neutralising solution. DNA was then transferred to a "Hybond-N" nylon membrane as described by Sambrook *et al.* (1989b) and subsequently cross-linked to membranes by UV irradiation for 5 mins. Membranes stored at 4°C until required.

Probe production. All experiments involving radiolabelled probes were performed in a designated radioisotope laboratory under conditions in accordance with University of London radioisotope handling guidelines.

Oligonucleotides were 5'-labelled to generate probes for hybridisation experiments by incubation of 5 pm oligonucleotide in 50 µl of T4 polynucleotide kinase reaction buffer containing 15 µl of 3,000 Ci/mmol [$\gamma^{32}\text{P}$]-ATP and 15 U of T4 polynucleotide kinase for 5 mins at 37°C. Reactions were subsequently terminated by the addition of 2 µl 0.5 M EDTA and stored at -20°C until required.

The percentage incorporation and specific activity of probes was determined by a filter-binding assay: 1 µl of reaction product was mixed with 100 µl 0.2 M EDTA and 3 µl of this solution spotted onto each of two pieces of Whatman 3MM filter paper. One piece was washed twice with 0.5 M NaPO₄, pH 7.4 for 5 mins to remove unincorporated [$\gamma^{32}\text{P}$]-ATP. Both filters were then air dried, added to separate scintillation vials containing scintillation fluid, and counts per minute (cpm) measured for each sample in a scintillation counter. The percentage incorporation was calculated as the incorporated cpm, divided by the total cpm multiplied by 100. Results indicated that the reactions had consistently

generated [$\gamma^{32}\text{P}$]-ATP incorporation efficiencies of over 80%.

The specific activity of the probe was calculated as the total cpm incorporated divided by the amount of labelled oligonucleotide expressed, as $\text{cpm}\mu\text{g}^{-1}$.

Hybridisation. All hybridisations were performed at 50°C. Nylon membranes were placed in a plastic container with a secure lid containing between 50 and 200 ml (depending on the size of the membrane) of prewarmed hybridisation buffer. This container was placed in an outer plastic box containing prewarmed water and the boxes placed in a water bath gentle shaking for 2 hrs. Following prehybridisation, the inner container was removed and 2 μl of oligonucleotide labelling reaction was added per 100 ml of hybridisation buffer. The container was replaced in the outer box and gently shaken at 50°C for 16 hrs.

Membranes were subsequently removed and washed twice in 100 ml of prewarmed wash buffer at 50°C for 10 mins each. Membranes were air dried, wrapped in cling film, placed in an autoradiography cassette, covered with Fuji RX X ray film (20x 30 cm) and placed at -70°C for 2 hrs. Film was processed automatically using a Kodak X-omat processor according to the manufacturer's instructions. If necessary, membranes were stored with X ray film for longer periods of time (up to several weeks) to allow for weak hybridisation signals to develop.

Membrane reprobng. To permit reprobng of membranes, hybridised membranes were stripped of probe by placing in a glass beaker containing a solution of 0.1% SDS and 0.1x SSC and boiling for 10 mins. Membranes were subsequently removed, air dried, wrapped in cling film, and autoradiographed. The absence of any image on the film was taken as an indication that all, or at least a significant proportion of the hybridised probe had been removed and that the membranes were suitable for reprobng

ELGA. For some studies, NASBA reaction products were analysed by an enzyme-linked gel assay method (van der Vliet *et al.* 1993) consisting of a liquid hybridisation with a HRP-labelled DNA probe (AB10 or U1A3) followed by polyacrylamide gel analysis in a mini-vertical electrophoresis system.

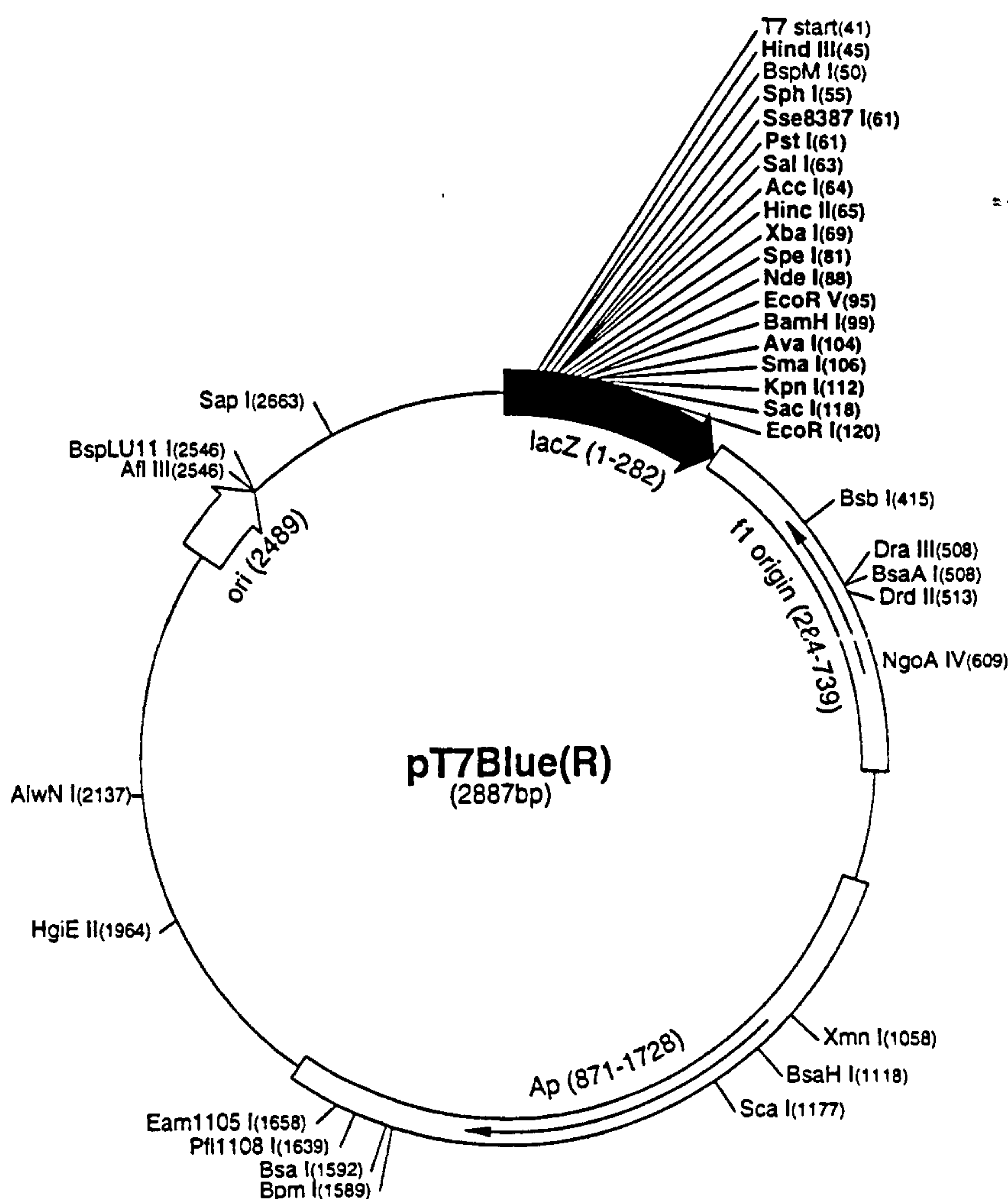
4.2.10. Cloning

All cloning procedures described in this project were performed in accordance with University of London genetic manipulation guidelines.

Three types of recombinant plasmid were produced in this study:

1. pT7Blue plasmids containing PCR products, or cDNA derived from NASBA products which were used in sequencing experiments.
2. pT7Blue plasmids containing MV1/MV2 PCR products - termed pT7BlueE - for the *in vitro* production of a measles RNA transcript which was used in experiments to quantify the sensitivities of measles RNA detection methods.
3. pT7BlueE plasmids containing a HIV1 gag1 DNA insert - termed pT7BlueE144 - used for the *in vitro* production of a modified measles RNA transcript which was used for competitive RT-PCR experiments.

Figure 4.1: pT7Blue; a T/A cloning vector



4.2.11. Insert preparation

NASBA products. NASBA reactions were deproteinised by phenol/chloroform/isoamyl alcohol separation and precipitated in isopropanol. Blunt-ended cDNA was prepared from 5 µg of product using a "Copy kit" according to the manufacturer's instructions (Gubler and Hoffman, 1983) and purified again by phenol/chloroform/isoamyl alcohol treatment and precipitation in 0.1 volume of 3M sodium acetate, pH 5.0, and 2.5 volumes of cold ethanol. cDNA was precipitated by incubation for 16 hrs at -20°C and recovered by centrifugation at 12,000 g for 15 mins at 4°C. The supernatant was discarded, cDNA pellets washed twice in 70% (v/v) ethanol and resuspended in 20 µl sterile ddH₂O. To check efficient isolation, cDNA was analysed by agarose gel electrophoresis and cDNA concentration was determined spectrophotometrically. ✓

5' termini of the blunt-ended cDNA ~~was~~ phosphorylated by incubation of 10 pm of purified cDNA in a 50 µl reaction containing 1x T4 polynucleotide kinase reaction buffer, 4 mM ATP and 15 U T4 polynucleotide kinase for 5 mins at 37°C. Reactions were terminated by the addition of 2 µl 0.5 M EDTA, reaction mixtures deproteinised, and phosphorylated products precipitated as described previously. ✓

PCR products. 20 µl of PCR product were electrophoresed at 100 volts in a 2% low melting point agarose gel containing 10 µgml⁻¹ ethidium bromide until the DNA fragment of interest was resolved. The gel was transferred to a UV transilluminator and a slice of gel containing the DNA fragment was removed with a sterile scalpel. The gel slice was transferred to a sterile 1.5 ml Eppendorf tube and melted by incubation at 70°C for 5 mins. DNA was separated from the melted gel using a "Wizard PCR preps DNA purification system" according to the manufacturer's instructions. DNA was eluted in 20 µl of sterile ddH₂O and analysed by agarose gel electrophoresis and its concentration determined spectrophotometrically.

HIV1 gag1 restriction fragment. 10 ng of a 144 bp *Pst*I / *Sph*I restriction endonuclease fragment from HIV1 gag1 DNA was made blunt-ended by its incubation with 5 U T4 DNA polymerase in a 20 µl reaction containing 1x T4

DNA polymerase reaction buffer, 100 μ M each dNTP and 0.1 mgml⁻¹ BSA for 5 mins at 37°C. Reactions were subsequently terminated by heating to 75°C for 10 mins. DNA was deproteinated, precipitated and recovered DNA resuspended in 20 μ l sterile ddH₂O.

4.2.12. Vector preparation

pT7BlueE. 1 μ g of pT7BlueE was linearised by incubation 10 U *Nsi*I in a 50 μ l reaction containing 1x reaction buffer for 2 hrs at 37°C. Reaction mixtures were deproteinised, DNA precipitated, and recovered DNA resuspended in 20 μ l of sterile ddH₂O. To ensure complete plasmid linearisation, plasmid was analysed by agarose gel electrophoresis, and DNA concentration was determined spectrophotometrically.

Linearised plasmid was blunt-ended using T4 DNA polymerase, reactions deproteinised and DNA recovered as described previously.

5' plasmid DNA termini were dephosphorylated by incubation with 1 U alkaline phosphatase per pmol 5' DNA ends in a 50 μ l reaction containing 1x alkaline phosphatase reaction buffer for 15 mins at 37°C, then 15 mins at 56°C. A further 1 U of alkaline phosphatase was added to the reaction and the incubations repeated.

pT7Blue. For the ligation of blunt-ended insert DNA, 500 ng pT7Blue was blunt-ended using T4 DNA polymerase as described above. DNA was purified, analysed and DNA concentration determined as described above.

4.2.13. Ligations

PCR products and pT7Blue. PCR products can be ligated directly with a T/A cloning vector (Marchuk *et al.* 1991). 100 ng of plasmid pT7Blue and an equal molar ratio of purified PCR product were ligated in a 10 μ l reaction composed of 1x T4 DNA ligase buffer and 1 Weiss unit of T4 DNA ligase. Reactions were incubated at 16°C for 16 hrs and stored at -20°C.

Blunt-ended cDNA and pT7Blue. 100 ng of phosphorylated, blunt-ended cDNA derived from NASBA reaction products were ligated with blunt-ended

pT7BlueE at an insert:vector molar ration of 3:1 as described previously.

HIV1 gag1 restriction fragment and pT7BlueE. 10 ng of purified, blunt-ended, dephosphorylated plasmid pT7BlueE and all of the recovered blunt-ended, phosphorylated HIV1 gag1 DNA fragment were ligated as described previously.

4.2.14. Transformation of competent *E.coli*

All transformations were performed in triplicate. 10^{10} "MAX efficiency" competent DH5 α *E.coli* cells were dispensed into 20 μ l aliquots in sterile 1.5 ml Eppendorf tubes. 1 μ l of ligation reaction mixture was added and the suspension mixed by gentle pipetting and left on ice for 30 mins. The suspension was shocked by placing at 42°C for exactly 40 secs then placed on ice for 2 mins. 80 μ l of sterile SOC media was added to the suspension and the mixture incubated with gentle shaking for 1 hr at 37°C.

50 μ l of the suspension was subsequently spread onto LB agar plates containing 50 μ gml $^{-1}$ ampicillin using a sterile glass rod and left to dry. Agar plates were incubated inverted at 37°C until the colonies developed.

Controls were included to determine the rate of spontaneous mutation to ampicillin-resistance, and for transformation efficiency.

4.2.15. Screening of transformants

pT7Blue possesses a functional LacZ operon (coding for β -galactosidase) for blue/white screening of recombinant plasmids. IPTG is an inducer of the LacZ operon while Xgal is a substrate for β -galactosidase which when cleaved, releasing a blue indolyl derivative. Bacterial colonies containing recombinant plasmids retain a white colouration while those containing no insert DNA appear blue.

Transformants containing pT7Blue vectors were screened by growth on LB agar plates containing Xgal and IPTG. Single white bacterial colonies were transferred to sterile 20 ml universal tubes containing 3 ml LB media and 50 μ gml $^{-1}$ ampicillin using sterile pipette tips. The suspensions were incubated for 16 hrs with shaking at 37°C to culture transformed *E.coli*.

To determine the efficiency of the ligation reactions, the blue bacterial colonies

(containing self-ligated plasmids) and the number of white bacterial colonies (containing recombinant plasmids) were counted following incubation of transformation reactions on agar plates containing Xgal and IPTG. The ligation efficiency was thus the number of white bacterial colonies divided by the total number of bacterial colonies.

As pT7BlueE did not possess a functional LacZ operon, transformed bacterial colonies were cultured, plasmid DNA isolated and screened for insert DNA by restriction digestion reactions described below.

4.2.16. Recombinant plasmid isolation and analysis

Plasmid isolation. Recombinant plasmids were isolated from bacterial culture using a "Wizard plus minipreps DNA purification system". Recovered plasmid DNA was precipitated in ethanol and the recovered DNA resuspended in 30 µl sterile ddH₂O.

Plasmid analysis. 1 µg of plasmid DNA was analysed for the presence of measles-specific insert DNA by the following restriction endonuclease digestions:

1. pT7BlueE vectors (pT7Blue vectors containing MV1/MV2 PCR product insert) by digestion with 10 U *SacI* and 10 U *NsiI* in a 20 µl reaction containing 1x reaction buffer at 37°C for 2 hrs.
2. pT7Blue vectors containing PCR products, or cDNA generated from NASBA products by restriction endonuclease digestion with 10 U *NsiI* in a 20 µl reaction containing 1x reaction buffer at 37°C for 2 hrs.
3. pT7BlueE vectors containing HIV-1 gag1 insert DNA by restriction digestion with 10 U *HindIII* in a 20 µl reaction containing 1x reaction buffer at 37°C for 2 hrs.

Products from restriction digestion reactions were analysed by agarose gel electrophoresis.

4.2.17. *In vitro* transcription

Template preparation. 10 µg of purified pT7BlueE or pT7BlueE144 were linearised downstream from the insert DNA sequence using 50 U *EcoRI* in a 50 µl reaction at 37°C for 2 hrs. 1 µl of digested plasmid was analysed by agarose gel electrophoresis to ensure efficient plasmid linearisation. Reaction mixtures were deproteinised, linearised plasmids precipitated in ethanol and recovered DNA resuspended in 20 µl of DEPC-treated ddH₂O. The concentration of recovered DNA was determined spectrophotometrically.

In vitro transcription. 2 µg of purified, linearised plasmid DNA were used as templates for *in vitro* transcription using a "T7 Transcription kit" according to the manufacturer's instructions. Post reaction, template DNA was removed by the addition of 10 U RNase-free DNaseI and the mixture incubated at 37°C for 15 mins. The reaction was stopped by the addition of 2 µl 0.5 M EDTA, deproteinised and transcript RNA recovered and stored as described in section 4.2.3.

Plasmid DNA contamination of transcripts. The presence of any contaminating plasmid DNA in the measles transcript RNA (e.g. from incomplete DNaseI digestion) was determined by performing PCR (using *Taq* DNA polymerase) without prior RT on a serial dilution of wild-type transcript. Primers MV3 and MV4 were used to amplify contaminating plasmid DNA in transcript RNA as described in section 4.2.5.

Additionally, RNA transcripts were pretreated with 0.05 µgml⁻¹ RNase A for 30 mins at 37°C. Products were amplified by PCR as above to further demonstrate the presence of contaminating plasmid DNA in transcript RNA samples.

4.2.18. DNA sequencing

DNA sequencing in this project was carried out by the method developed by Sanger (1977). All sequencing reactions were performed in duplicate and in both directions to ensure that accurate sequence data was obtained.

Gels. Two large sequencing glass plates were cleaned thoroughly with 70% (v/v) ethanol in ddH₂O. To prevent the gel sticking to the glass plates, 1 ml of dimethyldichlorosilane was spread over the surface of each plate using tissue paper. After 5 mins, tissues soaked in 70% ethanol were used to gently wipe away the dimethyldichlorosilane. Glass plates were assembled with spacers and sealed with tape to prevent leaking of the gel.

100 ml of "gel mix" containing 3 mM TEMED and 0.6% ammonium persulphate was made up according to the manufacturer's instructions, mixed thoroughly and poured between the two glass plates. After 30 mins, the glass plates and gel were placed in the sequencing apparatus, covered with 1x TBE and a shark-tooth comb positioned at the top of the gel.

Sequencing reactions. All experiments using [$\alpha^{35}\text{S}$]-dATP were performed in accordance with the University of London radioisotope handling guidelines.

1 μg of purified plasmid DNA obtained as described in section 4.2.13. was used as template for sequencing reactions using a "ThermoSequenase cycle sequencing kit" according to the manufacturer's instructions. Reactions contained [$\alpha^{35}\text{S}$]-dATP, one dideoxy NTP and either a forward (FP) or reverse (RP) sequencing primer (described in Table 4.8). Following cycle sequencing, 2 μl of stop dye was added to terminate the reactions which were stored at -20°C until required.

Gel analysis. Reactions products were denatured at 95°C for 5 mins and 3 μl loaded into the sequencing gel and electrophoresed at 1,600 volts for 3 hrs. Subsequently, the glass plates were separated and the gel still attached to one plate was carefully transferred to 2 l of fix solution (Table 4.4.). After gentle agitation for 30 mins at 20°C the gel and glass plate was removed from fix solution and a sheet of Whatman 3MM filter paper placed over the gel. residual fix solution was removed from the filter paper and the gel and filter paper was separated from the glass plate and covered in cling film. The gel was subsequently dried at 80°C under vacuum for 1hr then cooled to 20°C and placed in an autoradiography cassette with Fuji RX X ray film (30x 40 cm). The cassette was left for 24 hrs at 20°C and the film developed automatically as

described previously.

Automated DNA sequencing. As an alternative to manual DNA sequencing, 500 ng of purified plasmid DNA and gel-purified PCR products were sequenced using an ABI Prism (version 2.1.1.) automated DNA sequencer. This was carried out at the Microchemical Facility, Babraham Institute.

Chapter 5

Chapter 5: DEVELOPMENT OF AN ASSAY SYSTEM FOR USE IN EXPERIMENTS TO COMPARE THE SENSITIVITIES OF THREE NUCLEIC ACID AMPLIFICATION METHODS FOR MEASLES RNA DETECTION

5.1. INTRODUCTION

A region of the measles virus genome was cloned and used as a template for *in vitro* transcription. Measles RNA transcripts thus generated ~~was~~ used as templates for measles RNA amplification to aid in detection sensitivity experiments. In addition, these transcripts could be used to "spike" samples during future methods development experiments described in Chapter 8.

Three methods were used in this project for measles RNA detection: RT followed by nested PCR, using MMLV reverse transcriptase and *Taq* DNA polymerase; combined RT-PCR using *rTth* DNA polymerase; and NASBA. Experiments were conducted using the transcript generated above to assess their relative efficiencies of measles RNA detection in water. In addition, total RNA from a measles virus-infected cell line, and total RNA from a measles vaccine sample were also used to confirm the relative sensitivities of the three detection methods.

Aim. The aims of this part of the project were to:

1. Clone a region of the measles genome to permit the synthesis of measles RNA transcripts.
2. Determine the relative sensitivities of three nucleic acid amplification methods using a range of measles RNA templates derived from different sources.

5.2. RESULTS

5.2.1. *In vitro* measles N-gene transcript generation

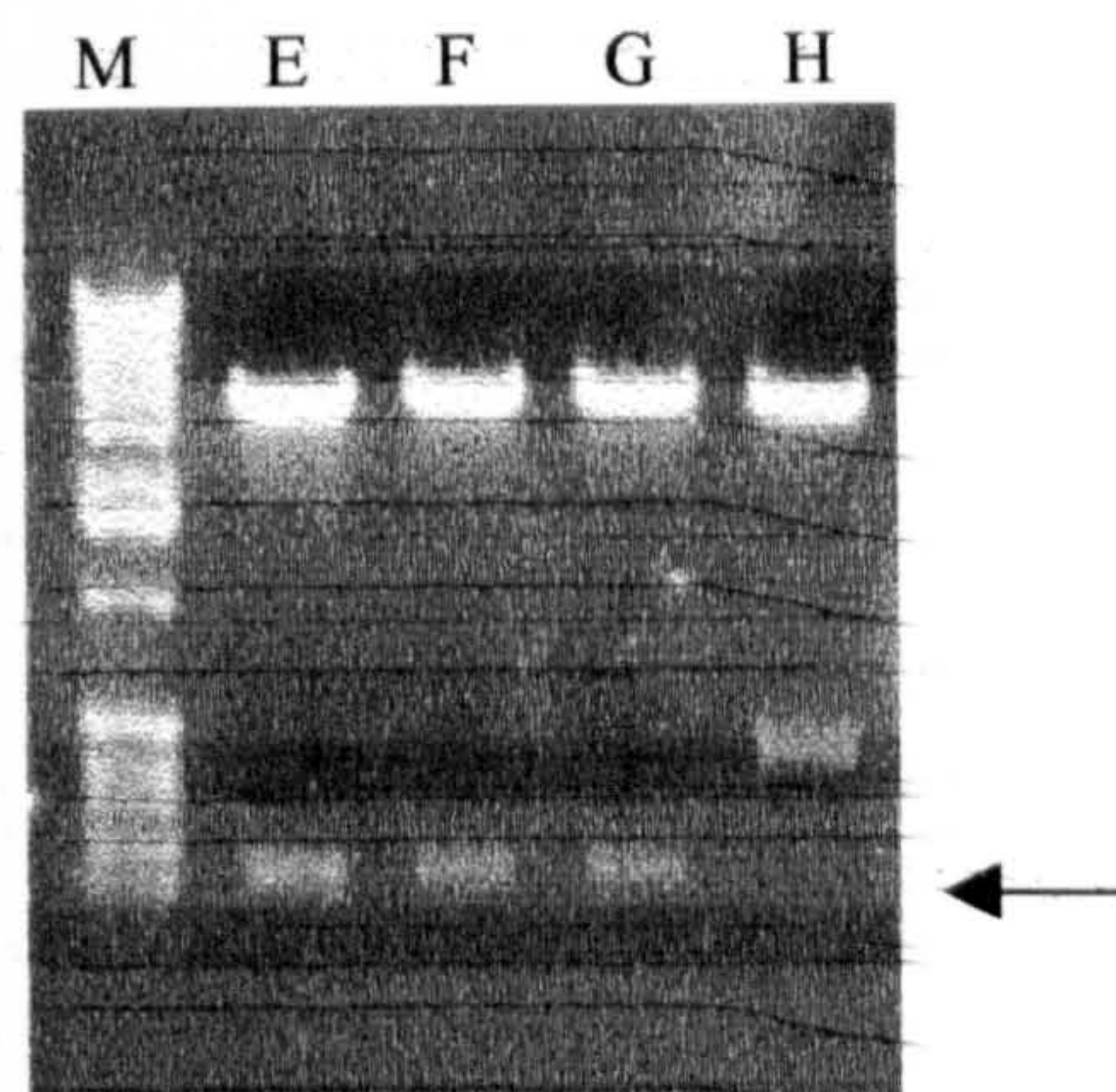
Cloning (Figure 5.1.). Total RNA was isolated from measles-infected Vero cells as described in section 4.2.3. 100 ng of isolated total RNA was used in an RT reaction with random hexamers and 1 µl of the product from RT used for PCR, with primers MV1 and MV2 and *Taq* DNA polymerase (section 4.2.5). The resulting 433 bp PCR product contained both measles PCR and NASBA primer binding sites and 50 ng of this PCR product was gel-purified and ligated with pT7Blue (section 4.2.10.).

Plasmids were transformed into competent *E.coli* (section 4.2.11.), and blue and white screening was used to select four transformed bacterial colonies containing recombinant plasmids (section 4.2.12.). Transformed bacterial colonies containing recombinant plasmids were cultured and recombinant plasmid DNA isolated (section 4.2.13.).

The presence of MV1/MV2 insert DNA, and its orientation within pT7Blue, was determined by double restriction endonuclease digestion of recombinant plasmid DNA with *SacI* and *NsiI* as described in section 4.2.13. The *NsiI* restriction site is unique to the MV1/MV2 insert DNA sequence, while the *SacI* restriction site flanks the insertion site on pT7Blue (Figure 4.1.). Therefore recombinant plasmids containing measles insert DNA in the MV1/MV2 orientation gave rise to a 323 bp restriction fragment, while recombinant plasmids containing measles insert DNA in the opposite (MV2/MV1) orientation gave rise to a 133 bp restriction fragment (Figure 5.1.).

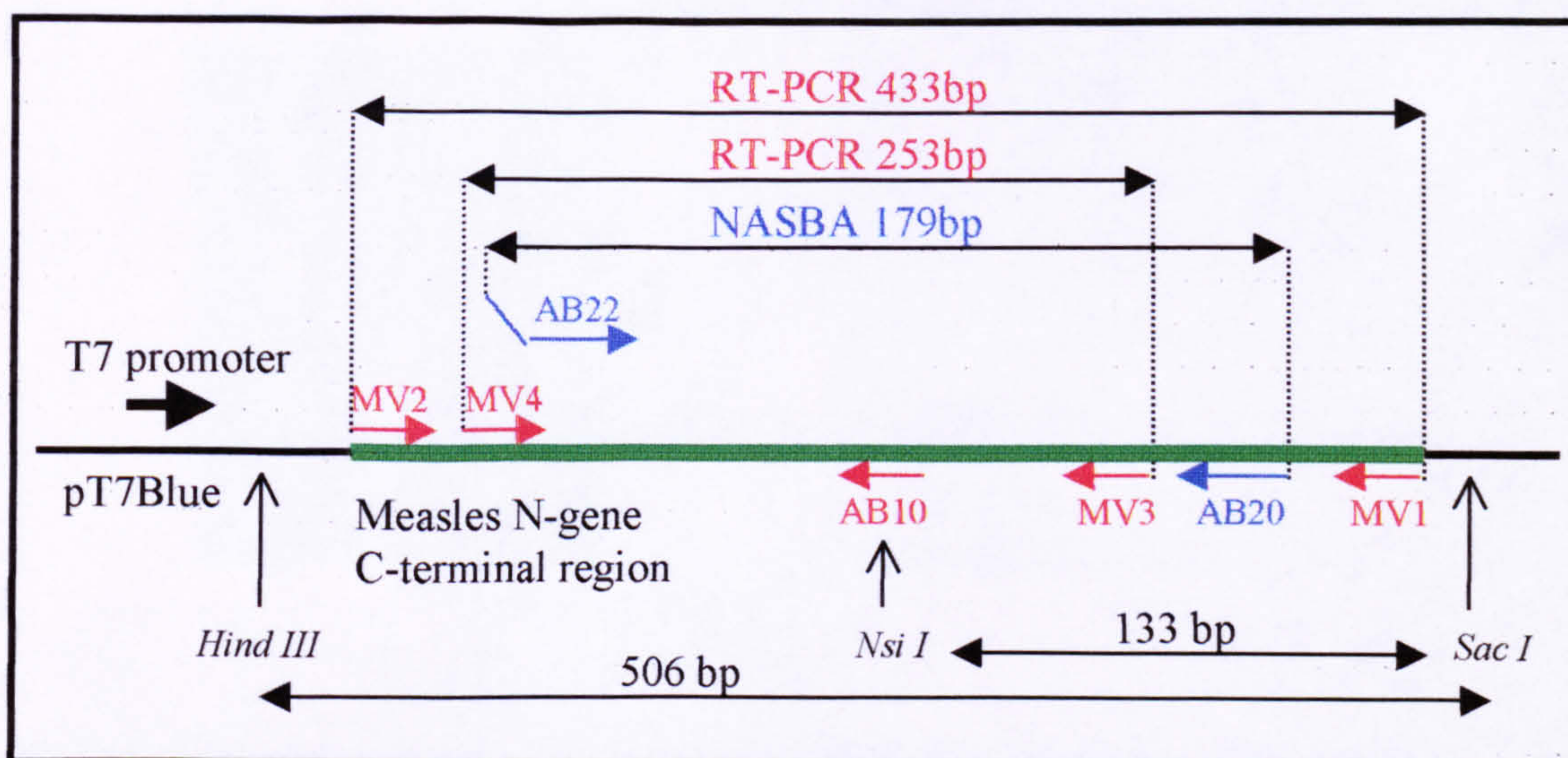
Recombinant pT7Blue vector from the transformed bacterial clone "E", containing measles DNA in the MV2/MV1 orientation was selected for *in vitro* transcription. A diagrammatic representation of pT7BlueE and measles amplification primer binding sites is given in Figure 5.2.

Figure 5.1: Restriction digests of recombinant pT7BlueE.



Recombinant plasmids E to H digested with *NsiI* and *SacI* to determine the specificity and orientation of insert DNA as described in section 4.2.13. The arrow points to a 133 bp DNA fragment indicating the presence of measles DNA in the MV2/MV1 orientation (plasmids E, F and G). Plasmid H contains insert DNA in the MV1/MV2 orientation giving rise to a 323 bp restriction fragment. Lane M contain a 1 kb DNA size marker described in Table 4.3.

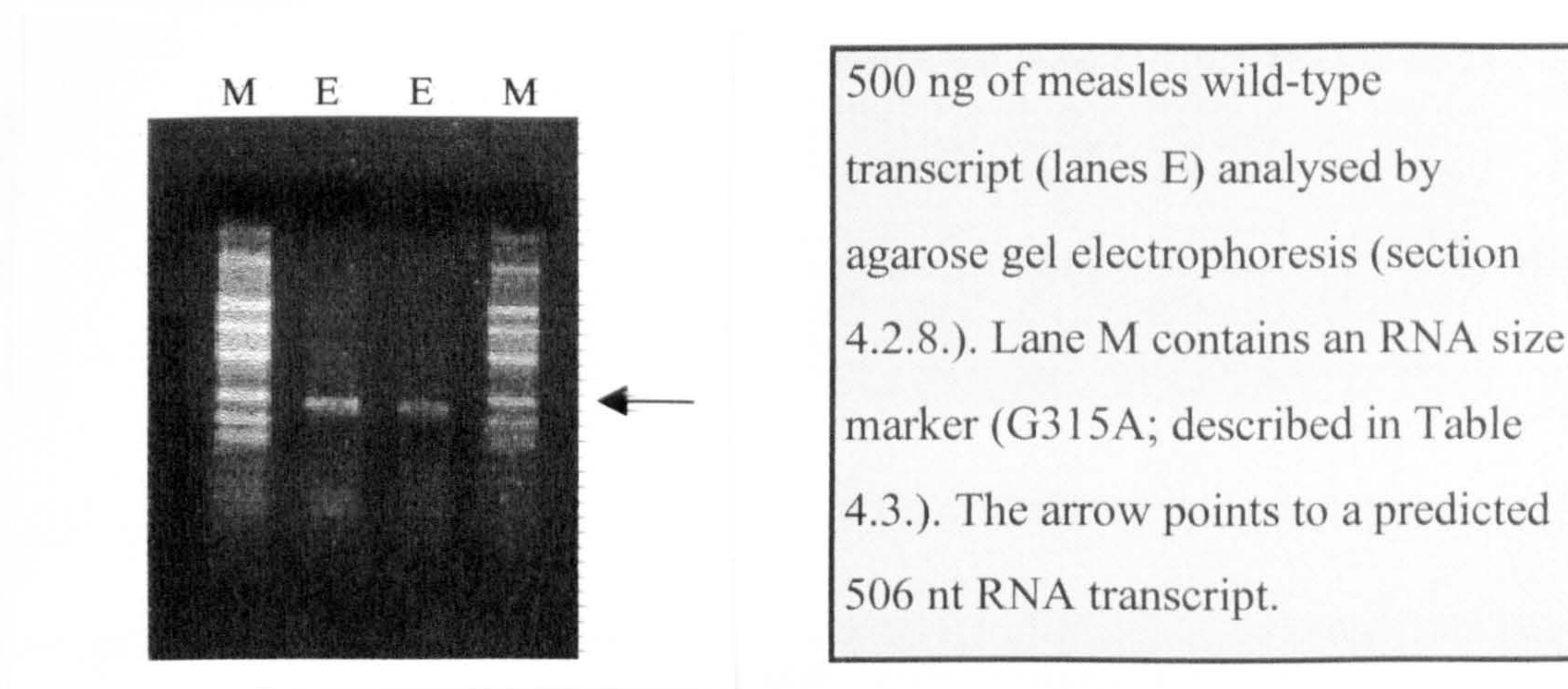
Figure 5.2: Measles amplification primer positions and relevant restriction sites on recombinant pT7BlueE.



In vitro transcription (Figure 5.3.). 10 μg of pT7BlueE was linearised downstream from the insertion site by restriction digestion with 50 U *EcoRI* (section 4.2.14.). 2 μg of linearised plasmid was recovered from this reaction and used as a template for *in vitro* transcription (section 4.2.14.).

Following *in vitro* transcription, plasmid DNA was removed by digestion with DNaseI and measles wild type RNA transcripts (506 nt in length) of negative sense were recovered from the mixture (section 4.2.14.). Transcript RNA was analysed by agarose gel electrophoresis (section 4.2.8.), its concentration determined spectrophotometrically (section 4.2.8.), and this concentration converted from $\text{ng}\mu\text{l}^{-1}$ to numbers of molecules μl^{-1} as the length of the RNA transcript was known. A stock serial dilution of transcript RNA was made up in water, dispensed into aliquots containing between 10^9 molecules and 1 molecule, and stored at -70°C until required.

Figure 5.3: Gel analysis of wild type measles RNA transcript.



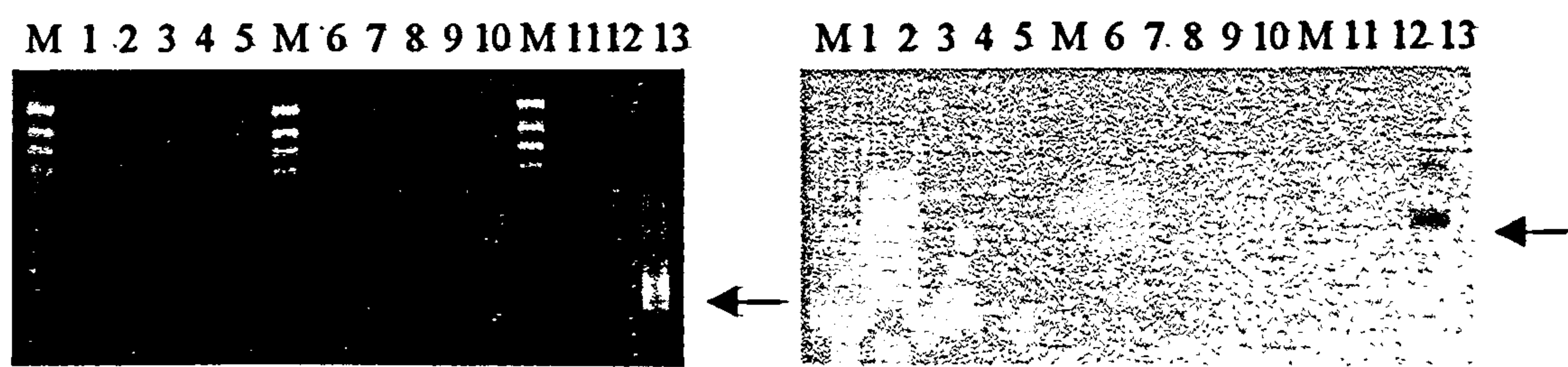
Detection of plasmid DNA contamination (Figure 5.4.). The presence of contaminating plasmid DNA in the recovered RNA transcripts may lead to an overestimation of the RNA transcript concentration. For this reason, it was important to check the measles RNA transcripts for contaminating plasmid DNA (section 4.2.14.).

PCR alone, using primers MV3 and MV5, was performed on the stock measles RNA transcript series. Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; section 4.2.9.).

PCR products were detected only when at least 10^9 transcript molecules were used as a template for amplification.

Next, PCR alone was performed on a serial dilution of RNA transcripts which had been pre-treated with RNase A to digest transcript RNA (section 4.2.14.). No PCR products were generated.

Figure 5.4: PCR analysis of transcripts for contaminating plasmid DNA.



a.

b.

(a.) Gel and (b.) Southern blot analysis of amplification products from a nested PCR reaction using *Taq* DNA polymerase with primers MV3 and MV5 (section 4.2.5.). Lanes 1 to 4 contain products from reactions with 10^9 to 10^6 measles transcripts pretreated with RNase and lanes 6 to 9 contain products from reactions with 10^9 to 10^6 measles transcripts without RNase pretreatment. Lanes 4, 10, 11, and 12 contain products from negative control reactions with water. Lane 13 contain products from a positive control reaction with cDNA from 1 ng measles-infected Vero cells. Southern blots were probed with radiolabelled AB10 (section 4.2.9.).

The arrow points to a 120 bp MV3/MV5 PCR product. Lane M contains a 100 bp DNA size marker.

5.2.2. Experiments to determine the relative sensitivities of RT-nPCR, *rTth*-mediated RT-PCR and NASBA.

RNA templates for measles amplification reactions. Three different measles RNA samples were used as templates. Firstly, measles wild type RNA transcripts synthesised from pT7BlueE. Secondly, total RNA isolated from Vero cells which had been infected with the Hu2 strain of measles virus (from a clinical isolate; Table 4.1.) as described in sections 4.2.2. and 4.2.3. Thirdly, total RNA isolated from four doses of a Schwarz strain measles vaccine sample (Table 4.1.) as described in section 4.2.3. In this latter case, the yield of total RNA was so low as to be unmeasurable spectrophotometrically and dilutions of vaccine RNA were quantified in terms of the dilution factor of the stock solution.

Amplification reactions.

1. RT-nPCR: RT, using random hexamers and MMLV reverse transcriptase, followed by nested PCR (primer pairs MV1 and MV2, then MV3 and MV4) with *Taq* DNA polymerase (section 4.2.5.).
2. *rTth*-mediated RT-PCR: using *rTth* DNA polymerase, primers MV3 and MV4 and EZ buffer (section 4.2.6.).
3. NASBA: using primers AB20 and AB22 (section 4.2.7.).

Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a ^{32}P -radiolabelled probe (AB10; section 4.2.9.).

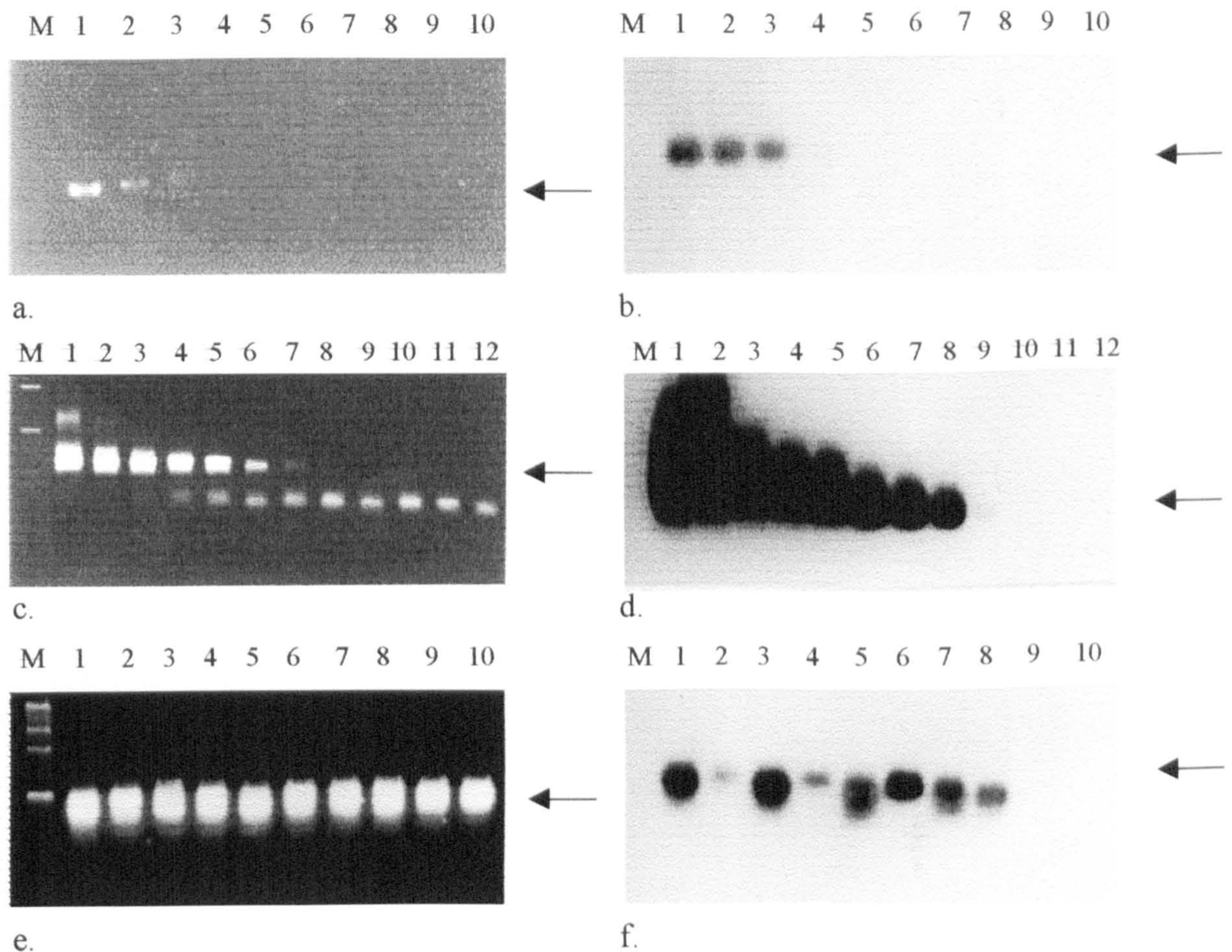
All experiments were performed at least three times to confirm data.

Measles wild type RNA transcript (Figure 5.5.).

1. RT-nPCR was observed to be able to detect as few as 10^8 copies of measles wild-type transcript RNA (Figure 5.5.a. and b.).
2. *rTth*-mediated RT-PCR was observed to be able to detect as few as 100 copies of the same transcript RNA (Figure 5.5.c. and d.).
3. NASBA was observed to be able to detect as ~~few~~ as 1 copy of this transcript RNA (Figure 5.5.e. and f.).

These sensitivities are represented in Table 5.1.

Figure 5.5: RT-nPCR, *rTth*-mediated RT-PCR and NASBA amplification of measles wild type transcript RNA.



(a.) Gel and (b.) Southern blot analysis (section 4.2.8.) of RT-nPCR products (section 4.2.5.) from a serial dilution from 10^{10} to 10^2 copies (lanes 1 to 9) of measles transcripts in water. Lane 10 contains products from a negative control reaction with water. The arrow points to the 253 bp MV3/MV4 PCR product.

(c.) Gel and (d.) Southern blot analysis of *rTth*-mediated RT-PCR products (section 4.3.6.) from a serial dilution from 10^{10} to 1 copies (lanes 1 to 11) of measles transcripts in water. Lane 12 contains products from a negative control reaction with water

(e.) Gel and (f.) Northern blot analysis of NASBA products (section 4.2.7.) from a serial dilution from 10^7 to 0 copies (lanes 1 to 9) of measles transcripts in water. Lane 10 contains products from a negative control reaction with water. The arrow points to a 179 nt AB20/AB22 NASBA product.

All reaction products were probed with radiolabelled AB10. Lanes labelled M contain a ϕ X174/*Hae III* DNA size marker, described in Table 4.3.

Total RNA from measles virus-infected Vero cells (Figure 5.6.).

- 1. RT-nPCR was observed to be able to detect measles RNA in as little as 10 pg total RNA from measles-infected Vero cells (Figure 5.6.a. and b.).
- 2. *rTth*-mediated RT-PCR was observed to be able to detect measles RNA in as little as 1 pg total RNA from measles-infected Vero cells (Figure 5.6.c. and d.).
- 3. NASBA was observed to be able to detect measles RNA in as little as 100 fg total RNA from measles-infected Vero cells (Figure 5.6.e. and f.).

These sensitivities are represented in Table 5.1.

Total RNA from a measles vaccine sample (data not shown).

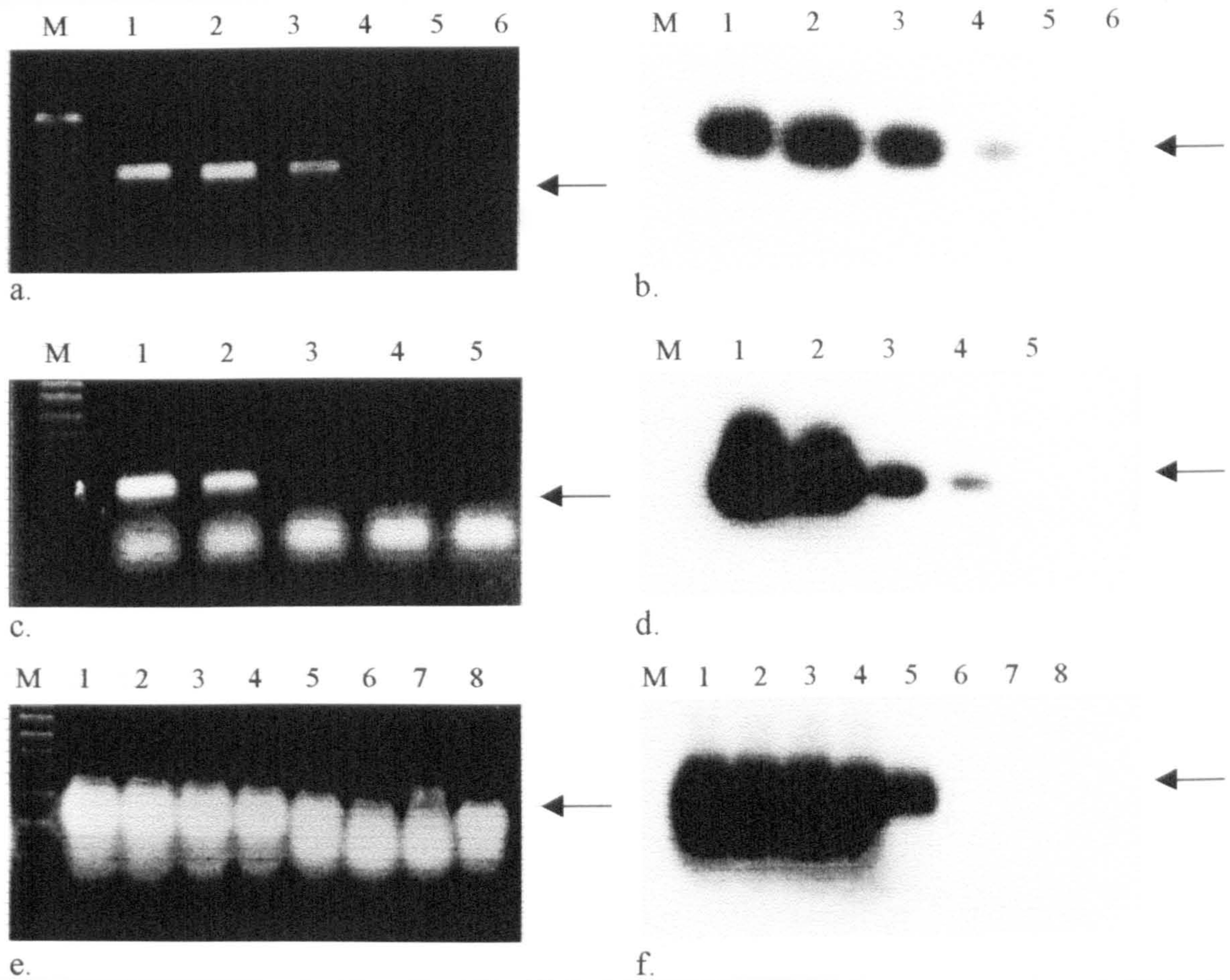
- 1. RT-nPCR was observed to be able to detect measles RNA in a 10⁻⁴ dilution of measles vaccine RNA.
- 2. *rTth*-mediated RT-PCR was observed to be able to detect measles RNA in a 10⁻⁵ dilution of measles vaccine RNA.
- 3. NASBA was observed to be able to detect measles RNA in a 10⁻⁶ dilution of measles vaccine RNA.

These sensitivities are represented in Table 5.1.

Table 5.1: The relative sensitivities of RT-nPCR, *rTth*-mediated RT-PCR and NASBA for measles virus RNA in water.

Measles RNA template	RT-nPCR	<i>rTth</i> -mediated RT-PCR	NASBA
Total RNA from measles- infected Vero cells	10 pg	1 pg	100 fg
Measles wild type RNA transcript	10 ⁸ molecules	10 molecules	1 molecule
Total RNA isolated from a measles vaccine sample	10 ⁻⁴ dilution	10 ⁻⁵ dilution	10 ⁻⁶ dilution

Figure 5.6: Amplification of total RNA from measles-infected Vero cells



(a.) Gel and (b.) Southern blot analysis (section 4.2.8.) of RT-nPCR products (section 4.2.5.) from a serial dilution from 10 ng to 1 pg (lanes 1 to 5) of total RNA from measles-infected Vero cells in water. Lane 6 contains products from a negative control reaction with water. The arrow points to a 253 bp MV3/MV4 PCR product. Lane M contains a ϕ X174/*Hae III* DNA size marker.

(c.) Gel and (d.) Southern blot analysis of *rTth*-mediated RT-PCR (section 4.2.6.) products from a serial dilution from 1 ng to 100 fg (lanes 1 to 5) of total RNA from measles-infected Vero cells in water. Lane 6 contains products from a negative control reaction with water.

(e.) Gel and (f.) Northern blot analysis of NASBA products (section 4.2.7.) from a serial dilution from 1 ng to 1 fg (lanes 1 to 7) of total RNA from measles-infected Vero cells. Lane 8 contains products from a negative control reaction with water. The arrow points to a 179 nt AB20/AB22 NASBA product. Lane M contains a 100 bp DNA size marker (Table 4.3.).

All reaction products were probed with radiolabelled AB10 (section 4.2.8.).

5.3. DISCUSSION

Cloning experiments successfully generated recombinant plasmids containing a region of the measles virus genome. One plasmid (pT7BlueE) was selected for *in vitro* transcription which contained insert DNA in the MV2/MV1 orientation. pT7BlueE yielded measles RNA transcripts representing the genomic, negative sense. Such negative sense measles RNA was an appropriate template with which to assess the efficiencies of nucleic acid amplification methods as negative strand genomic measles RNA is most likely to be detected in clinical samples persistently infected with measles virus, rather than positive strand, messenger RNA which is indicative of a replicating virus infection.

pT7BlueE was used to generate a measles wild type RNA transcript which was subsequently used to accurately quantify the sensitivity of various measles RNA detection methods. In addition, this transcript was used in later studies (Chapter 8) to spike samples in order to measure the efficiency of measles RNA isolation and amplification from clinical samples.

PCR alone on the transcript stock dilution series yielded specific products when 10^9 transcripts were used for amplification. However, these products may have arisen as a result of the reverse transcriptase activity of *Taq* DNA polymerase (Grabko *et al.* 1996), rather than from contaminating plasmid DNA. A subsequent experiment yielded no PCR products when transcript RNA was first digested with RNase A, indicating that the PCR products generated using at least 10^9 transcripts had probably been due to the reverse transcriptase activity of *Taq* DNA polymerase rather than the presence of contaminating plasmid DNA.

Total RNA was successfully isolated from measles-infected Vero cells and from a measles vaccine sample. This meant that three measles RNA templates were available to test the relative sensitivities of each measles RNA amplification method.

From experiments conducted to assess the relative efficiencies of the three methods to detect measles RNA in water, the results indicated that NASBA was the most sensitive method. NASBA showed at least a ten-fold increase in sensitivity over RT-nPCR and *rTth*-mediated RT-PCR (Table 5.1.).

The increased sensitivity of NASBA over RT-PCR may have been due to the

fact that NASBA is a continuous amplification process and therefore represents a more efficient method of RNA amplification compared to a two-step RT-PCR process.

These experiments represent the first time that NASBA has been used for the amplification of measles RNA.

The results of the detection sensitivity experiments showed at least a ten-fold increase in the sensitivity of measles RNA detection using *rTth*-mediated RT-PCR, compared to RT-nPCR for all the measles RNA templates analysed. This increase in sensitivity was particularly evident when the measles wild type transcript was used as a template for amplification. Such an increase could be due to the fact that elevated temperature (60°C) is used for reverse transcription when employing *rTth* DNA polymerase and EZ buffer, compared to MMLV reverse transcriptase (37°C). At this increased temperature RNA secondary structures could be reduced allowing more efficient reverse transcription. However, no increase in the detection sensitivity of RT-PCR using MMLV and *Taq* DNA polymerase was observed when RNA samples were denatured by heating prior to reverse transcription (data not shown).

The results generated in this part of the study indicate that the methods are a sensitive and potentially useful way of detecting measles virus RNA.

Chapter 6

Chapter 6: EXPERIMENTS TO DETECT MEASLES VIRUS RNA IN IBD TISSUE USING NASBA

6.1. INTRODUCTION

As a consequence of the results obtained from the previous study, NASBA was used in a preliminary study to analyse intestinal resection tissue from IBD patients for the presence of measles RNA. Full-thickness resection specimens were used for this study as they represented the most likely source of Crohn's disease granulomas - the location of measles antigen reported in previous studies (Wakefield *et al.* 1993 and 1997; Miyamoto *et al.* 1995; Lewin *et al.* 1995; Ekbohm *et al.* 1996 and Daszak *et al.* 1997).

In addition to IBD tissue, NASBA was used to analyse intestinal resection tissue from non-IBD control patients in order that the statistical significance of any measles RNA detected in IBD tissue could be determined.

To assess isolated total RNA integrity, NASBA for a housekeeping RNA species, U1A RNA - forming part of the U1A spliceosome subunit (Sillekens *et al.* 1987) - was performed. In addition, total RNA isolated from tissue samples was analysed by agarose gel electrophoresis. RNA samples which tested positive by U1A NASBA were used for measles NASBA reactions.

As a positive control for the ability of NASBA to detect measles RNA in persistently infected tissue, NASBA was performed on total RNA isolated from autopsy material of patients with SSPE.

To reduce the possibility of the generation of false positive results through laboratory contamination, the Norwood lab (University of Greenwich, London) was used for total RNA extraction and NASBA reactions, while laboratory 10.324 (Royal Free Hospital, London) was used for NASBA product analysis.

Aims. The aims of this part of the study was to:

well

1. Isolate total RNA from intestinal resection tissue and assess its integrity.
2. Confirm the ability of NASBA to detect measles RNA in persistently infected, SSPE tissue.
3. Use NASBA in a preliminary study to determine the presence of measles RNA in total RNA isolated from either IBD or control intestinal tissue.

6.2. RESULTS

6.1.1. Isolation of total RNA and assessment of RNA integrity.

Tissue samples and RNA isolation. Fresh-frozen intestinal resection tissue was obtained from 33 adult patients with Crohn's disease, 8 patients with ulcerative colitis and 20 non-IBD control patients. Post-resection time was below five minutes in all cases and samples had been stored at -70°C for no longer than five years. Tissue samples were coded prior to analysis.

SSPE autopsy brain material was supplied by the National Neurological Research Specimen Bank (Los Angeles, USA). The time between death and freezing of brain tissue (autolysis time) was up to 24 hrs. Three of the four SSPE samples were measles antigen positive as determined by immunohistochemistry (described elsewhere; Wakefield *et al.* 1997; data not shown). Details of SSPE tissue are given below.

Total RNA was isolated from approximately 500 mg of coded tissue (4.2.3.), and its concentration determined spectrophotometrically (section 4.2.8.).

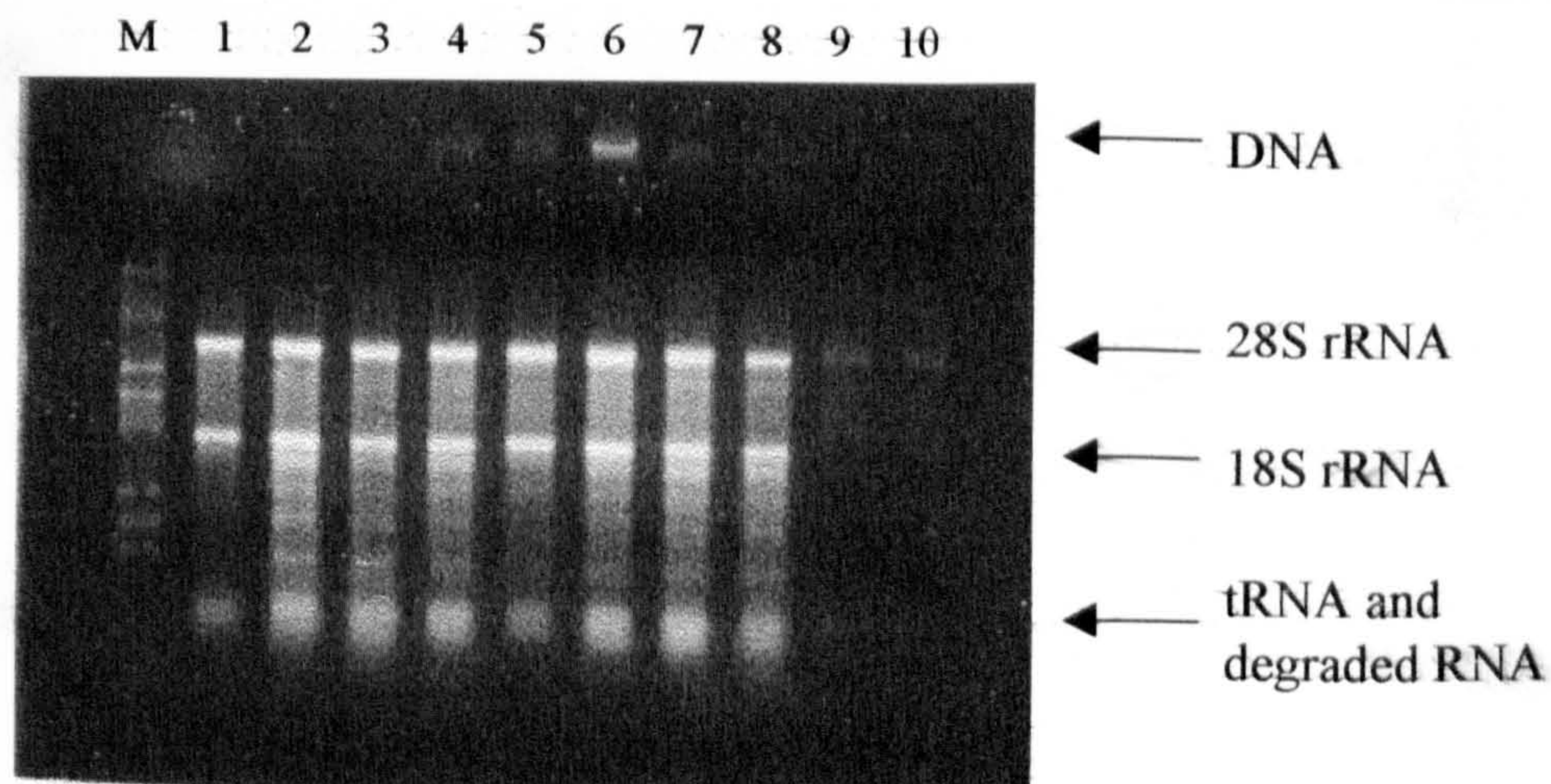
Table 6.1: Details of SSPE tissue (N/A not available).

SSPE sample	Age of patient (yrs)	Tissue autolysis time (hrs)
1	21	N/A
2	11	5
3	14	8
4	6	24

Integrity of total RNA isolated from tissue samples (Figures 6.1. and 6.2.). The integrity of total RNA isolated from tissue samples was assessed by agarose gel electrophoresis (section 4.2.8.) and NASBA for U1A RNA (section 4.2.7.). Following electrophoresis of 2 μ g of total RNA isolated from intestinal tissue, 28S and 18S ribosomal bands were clearly visible.

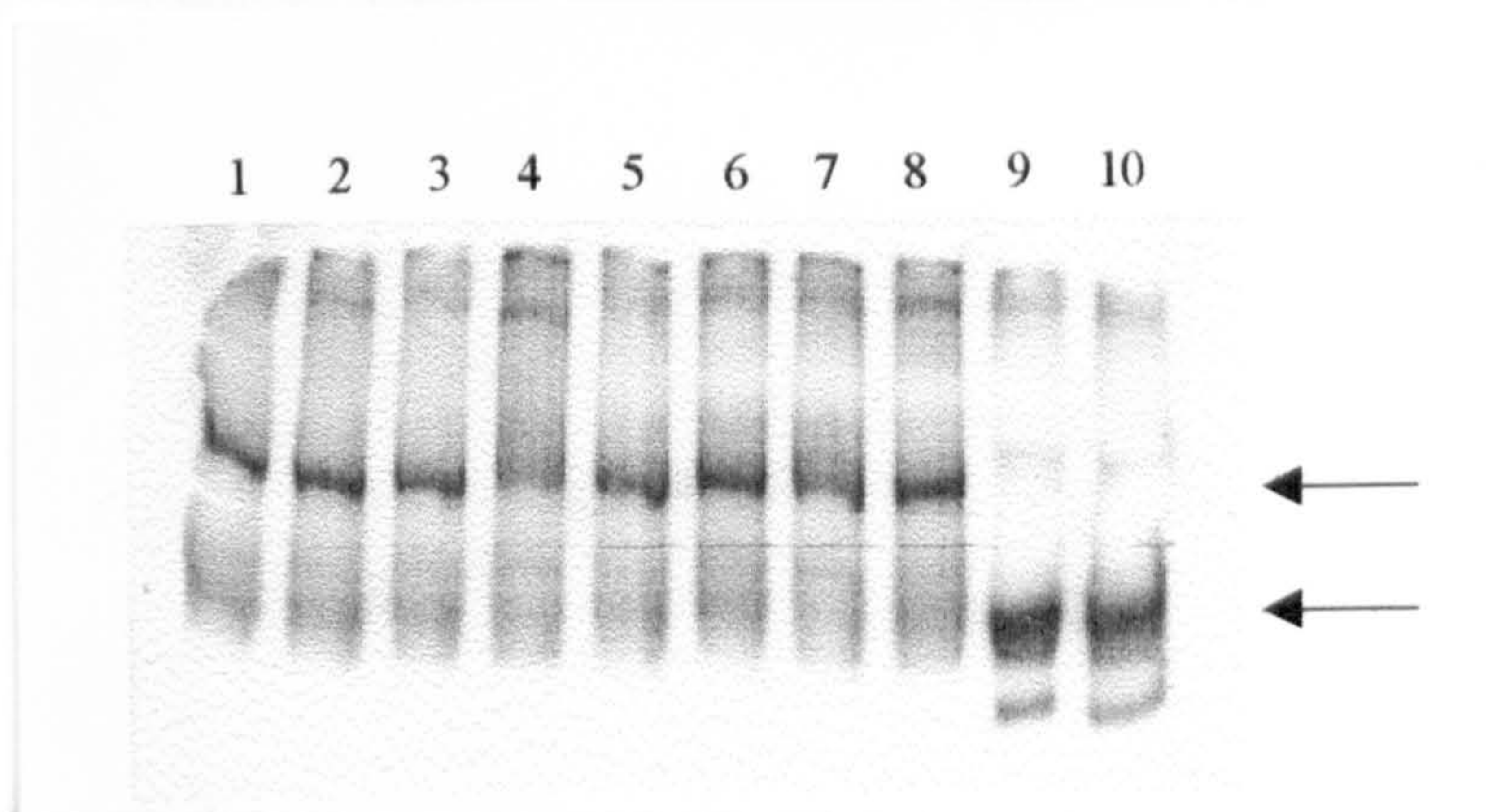
100 ng of total sample RNA was used for NASBA amplification of U1A RNA and reaction products analysed by ELGA using a HRP-labelled probe (U1A3; section 4.2.9). 59 of the 61 intestinal RNA samples tested positive for U1A RNA. All four SSPE samples were U1A NASBA positive (data not shown).

Figure 6.1: Gel analysis of total RNA isolated from intestinal tissue.



Representative agarose gel analysis (section 4.2.8.) of 2 μ g of total RNA prepared from intestinal resection tissue (lanes 1 to 8). Lanes 9 and 10 contain 500 ng total RNA from Vero cells as RNA standards. Lane M contains an RNA size marker (0.24 to 9.5 nt; Table 4.3.).

Figure 6.2: ELGA analysis of NASBA products.



Representative ELGA analysis of U1A NASBA products (section 4.2.8.). Lanes 1 to 8 contained 100 ng total sample RNA in NASBA reactions and lanes 9 and 10 contain products from a negative control reaction with water. Reaction products were probed with HRP-labelled U1A3 (section 4.2.9).

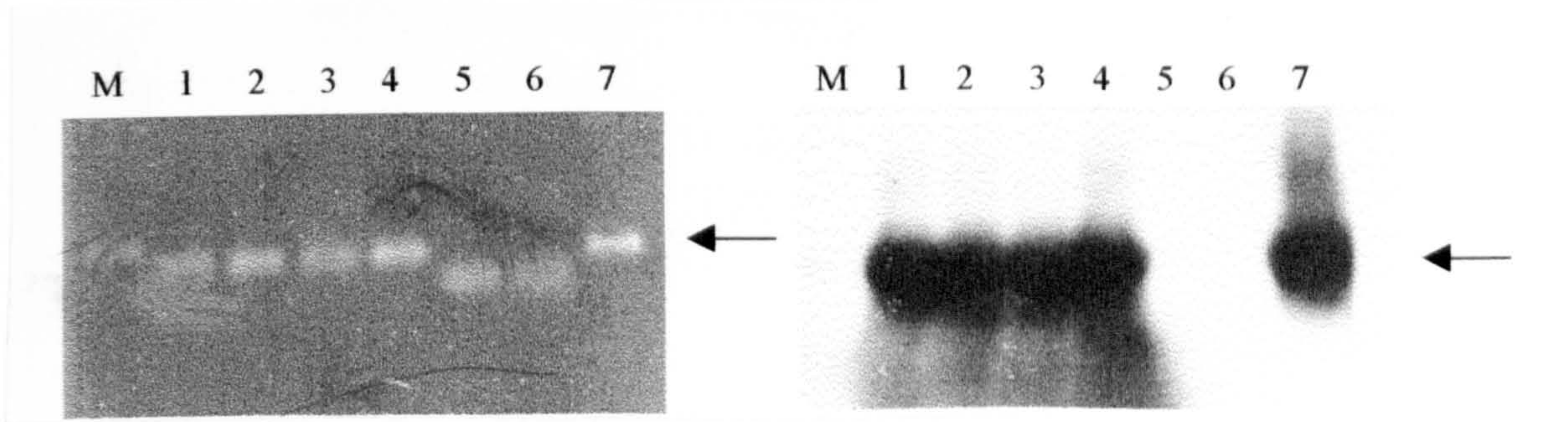
The upper arrow points to probe hybridised with U1A NASBA products while the lower arrow points to unhybridised probe.

6.2.2. Detection of measles RNA in SSPE RNA using NASBA.

Measles NASBA was performed using 100 ng of total RNA isolated from SSPE tissue specimens with primers AB20 and AB22 (section 4.2.7.). Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; section 4.2.9.).

All four SSPE samples yielded positive results following measles NASBA.

Figure 6.3: Analysis of total RNA from SSPE tissue for measles RNA using NASBA.



a.

b.

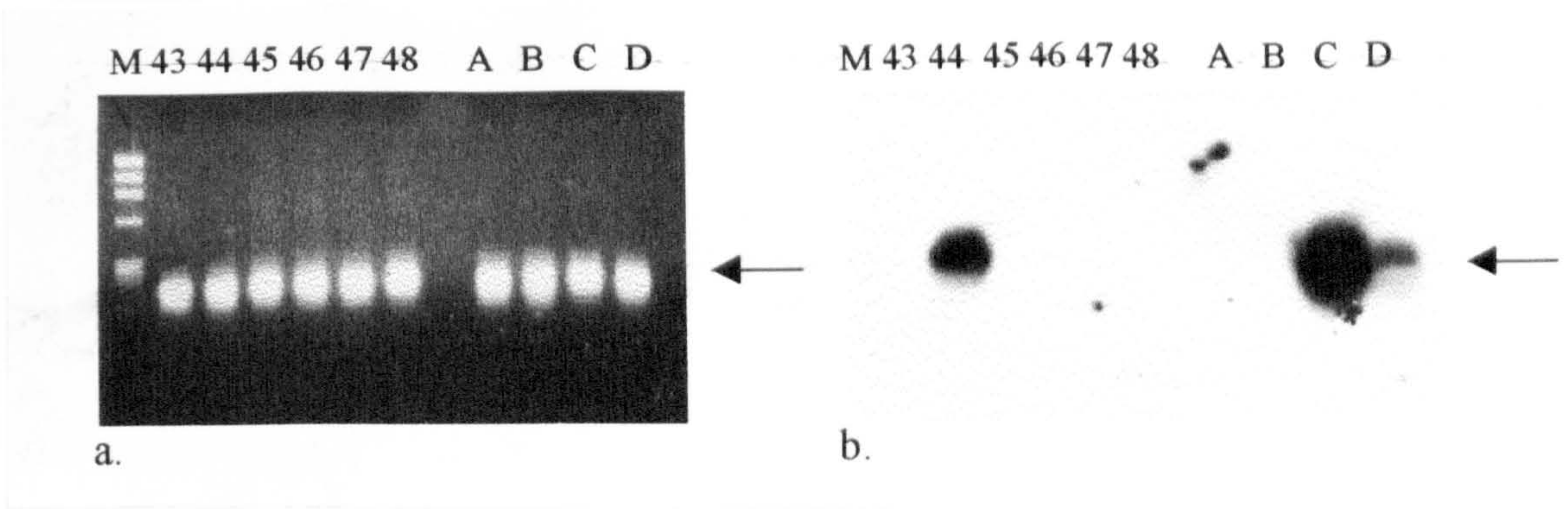
(a.) Gel and (b.) Northern blot analysis of NASBA products (section 4.2.7.) from total RNA isolated from SSPE autopsy material (lanes 1 to 4). Negative control reactions 5 and 6 contain water. Lane 7 contains products of a positive control reaction with 1 ng total RNA from measles virus-infected Vero cells. Northern blots were probed with radiolabelled AB10 (section 4.2.9.). Lane M contains an RNA size marker (0.24 to 9.5 nt; described in Table 4.3.).

6.2.3. Detection of measles RNA in intestinal RNA using NASBA.

Measles NASBA was performed using 100 ng of total RNA isolated from the 59 intestinal tissue specimens which had tested positive for U1A RNA, with primers AB20 and AB22 (section 4.2.7.). Reaction products were analysed either by ELGA, using an HRP-labelled probe (AB10; section 4.2.9.), or by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; section 4.2.9.).

Two of the 59 RNA samples (samples 9 and 44) yielded positive results following measles NASBA. The measles NASBA result for sample 44 is shown in Figure 6.4. After decoding, these samples were found to have originated from non-IBD control patients.

Figure 6.4: Analysis of total RNA from intestinal tissue for measles RNA using NASBA.



(a.) Gel and (b.) Northern blot analysis of NASBA reaction products (section 4.2.7.) from intestinal RNA samples 43 to 48. Lanes A and B contain products from negative control reactions with water. Lanes C and D contain products from positive control reactions with 10 pg and 1 pg, respectively, of total RNA from measles virus-infected Vero cells. Reaction products were probed with radiolabelled AB10 (section 4.2.9.). Lane M contains an RNA size marker (0.24-9.5 nt; Table 4.3.).

6.3. DISCUSSION

In experiments to determine the integrity of isolated total RNA, 28S and 18S ribosomal RNA bands were clearly visible following agarose gel analysis, indicating the isolation of good quality, undegraded RNA. Moreover, NASBA for low copy number U1A RNA confirmed this for all but two of the 61 intestinal resection tissues, and all four SSPE tissue samples.

Measles NASBA experiments using SSPE RNA yielded measles-specific amplification products, confirming the ability of NASBA to detect measles virus RNA in persistently infected tissue. Indeed, measles RNA was detected in one tissue where measles antigen had not been detected, emphasising the efficiency of the NASBA method.

In experiments to determine the presence of measles RNA in intestinal tissue using NASBA, measles RNA was detected in two non-IBD control tissues (samples 9 and 44) but not in any of the IBD tissue samples analysed.

It is possible that the positive results generated from intestinal samples 9 and 44 were the result of contamination of measles NASBA reactions with the laboratory Hu2 strain of measles virus. To exclude this possibility, it is necessary to sequence the measles NASBA products from these samples and compare the sequences with the sequence of the Hu2 measles virus strain. Such sequencing studies also provide information concerning the strain of measles virus present in these tissue samples.

The lack of detectable measles virus RNA in IBD tissue in this study may have been due to the sensitivity of the detection method employed. Although NASBA was found to be highly sensitive at detecting measles virus RNA diluted in water, the efficiency of measles RNA isolation and amplification from intestinal tissue homogenates was not determined and further studies are necessary to optimise the detection of low copy-number measles RNA in intestinal tissue.

Chapter 7

Chapter 7. A COMPARISON OF THE RELATIVE FIDELITIES OF RT-nPCR, *rTth*-MEDIATED RT-PCR AND NASBA AS SOURCES OF DNA FOR SEQUENCING

7.1. INTRODUCTION

a In order to confirm the specificity of a diagnostic amplification method, and to characterize its products, cloning and sequencing of such products is often employed. In addition, cloned DNA products from amplification reactions may be required for *in vitro* transcription and translation studies and for long-term storage.

Also, sequencing cloned amplification products of viral RNA or DNA templates enables the sequence variation within a viral RNA or DNA sample to be determined. This can provide valuable information concerning the quasispecies distribution within a given infected clinical sample (Ni et al. 1997).

However, enzymes involved with nucleic acid amplification can generate errors through misincorporation of incorrect bases into products (Roberts *et al.* 1989; Ling *et al.* 1991; Yu *et al.* 1992). Therefore multiple clones are required to be sequenced in order to obtain an accurate consensus sequence of a particular DNA or RNA product.

NASBA generates a majority of single-stranded RNA products which must first be converted to double stranded DNA to permit cloning. This can be achieved either by reverse transcription followed by synthesis of a second DNA strand, or by RT-PCR.

Aims. The aims of this study were to:

1. Generate plasmid clones of RT-nPCR, *rTth*-mediated RT-PCR and NASBA reaction products
2. Compare the fidelities of the three methods by sequencing cloned reaction products.
3. To sequence the measles NASBA products generated in the study described in the previous chapter.

7.2. RESULTS

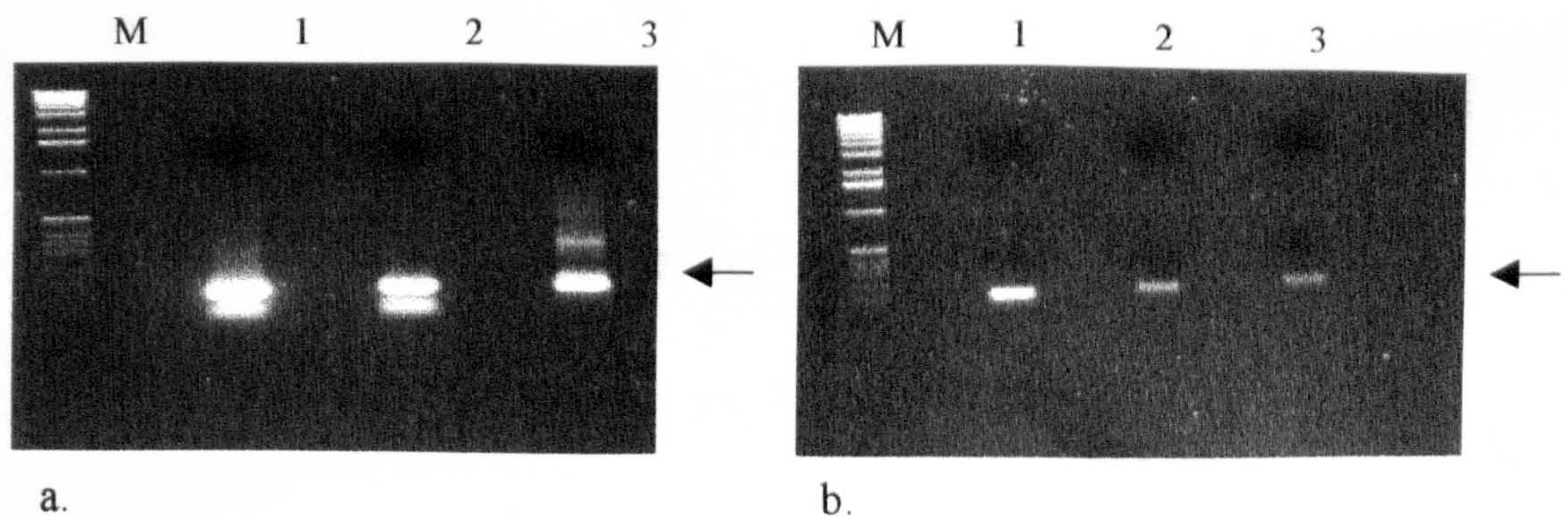
7.2.2. Generating plasmid clones of RT-nPCR, *rTth*-mediated RT-PCR and NASBA reaction products

Amplification reactions. Total RNA was isolated from measles virus-infected Vero cells (4.2.3.) and 100 ng of this isolated RNA was used as a template for the following measles RNA amplification reactions:

1. RT-nPCR: RT, using random hexamers and MMLV reverse transcriptase, followed by nested PCR (primer pairs MV1 and MV2, then MV3 and MV5) with *Taq* DNA polymerase (section 4.2.5.).
2. *rTth*-mediated RT-PCR: using primers MV3 and MV5 *rTth* DNA polymerase and EZ buffer (section 4.2.6.).
3. NASBA: using primers AB20 and AB22 (section 4.2.7.).

Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; section 4.2.9.).

Figure 7.1: Gel-purification of RT-PCR products



(a.) Gel analysis of *rTth*-mediated RT-PCR products (lanes 1, 2 and 3; section 4.2.6.) from reactions containing total RNA from measles-infected Vero cells.
(b.) Gel analysis DNA gel purified from the above reaction (section 4.2.11.).
The arrow points to the 120 bp MV3/MV5 DNA fragment.
Lane M contains a 1 kb DNA size marker (Table 4.3.).

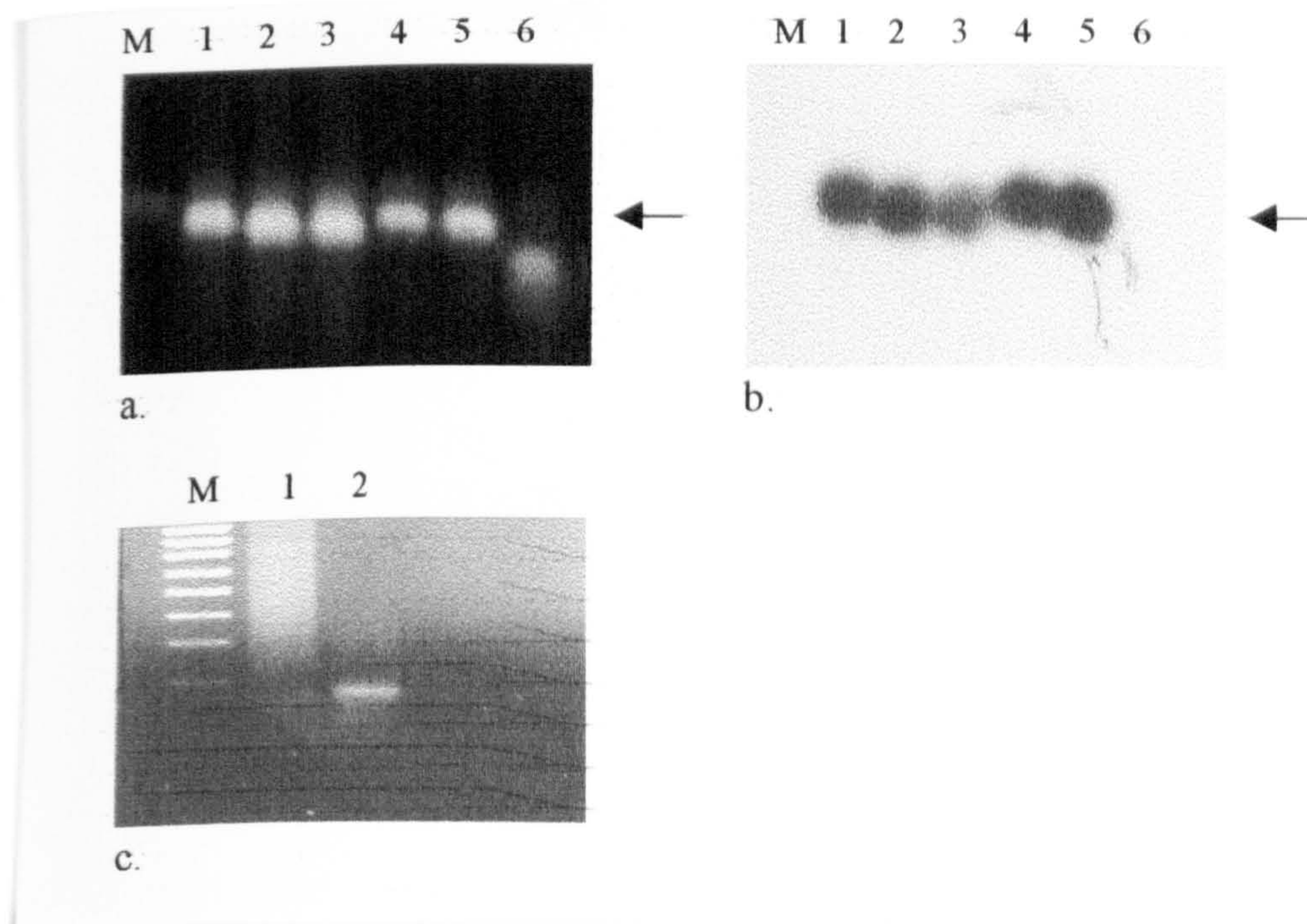
Generating double-stranded DNA from NASBA reaction products (Figure 7.2.).

NASBA reactions were deproteinised and recovered by isopropanol precipitation (section 4.2.11.) prior to the generation of double-stranded DNA.

Two strategies were employed to convert single-stranded RNA NASBA products to double-stranded DNA for cloning and sequencing. Firstly, 5 µg of purified NASBA product was used to generate blunt-ended, double stranded cDNA using a "Copy kit" (section 4.2.8.). cDNA products were analysed by agarose gel electrophoresis (section 4.2.8.).

Secondly, 1 ng of purified NASBA products were used in an *rTth*-mediated RT-PCR reaction containing primers MV3 and MV5 (section 4.2.6.). and products identities confirmed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; section 4.2.9.).

Figure 7.2: Generation of blunt-ended cDNA from NASBA products



(a.) Gel and (b.) Northern blot analysis of 5 μ l NASBA products (section 4.2.7.) generated from total RNA from measles-infected Vero cells (lanes 1 to 5). Negative control reaction 6 contained water. Reaction products were probed with radiolabelled AB10 (section 4.2.9.).

The arrow points to a 120 nt AB20/AB22 NASBA product. Lane M contains an RNA size marker (0.24-9.5 nt; Table 4.3.).

(c.) Gel analysis of cDNA generated from the above NASBA products (Lane 1) using a "Copy kit" (section 4.2.11.). Lane 2 contains cDNA generated from HeLa mRNA which is supplied as a positive control supplied with the kit. Lane M contains a 100 bp DNA size marker (Table 4.3.).

Cloning RT-PCR products. RT-nPCR and *rTth*-mediated RT-PCR reaction products were gel purified (section 4.2.8; Figure 7.1.) before cloning and 10 ng of purified product from both reactions directly ligated with pT7BlueE using T4 DNA ligase (section 4.2.10). Ligation products were transformed into competent *E.coli* (section 4.2.11.) and recombinant plasmids identified using blue and white screening (section 4.2.12.). Ten transformed bacterial colonies containing recombinant plasmids were selected for culture and plasmid DNA was isolated from these cultures (section 4.2.13.).

Cloning cDNA generated from NASBA products. cDNA generated from NASBA products was blunt-end cloned into pT7BlueE. 10 pmol of blunt-ended cDNA generated from NASBA products was phosphorylated using T4 polynucleotide kinase (section 4.2.8.). 100 ng of pT7Blue plasmid DNA was blunt ended with T4 DNA polymerase and dephosphorylated with alkaline phosphatase (section 4.2.9.).

DNAs recovered from these reactions were ligated using T4 DNA ligase (section 4.2.10.). and ligation mixtures used to transformed competent *E.coli* (section 4.2.11.). 24 transformed bacterial colonies were selected for culture and plasmid DNA isolated from these cultures (section 4.2.13.). Plasmids were screened for the presence of measles insert DNA by digestion with *NsiI* (section 4.2.13.). Ten plasmid clones containing measles insert DNA were selected for sequencing experiments.

7.2.2. Fidelities of RT-nPCR, *rTth*-mediated RT-PCR and NASBA reactions.

Sequencing. Recombinant plasmids containing cloned measles insert DNA were sequenced manually in forward and reverse directions using a "Thermo-Sequenase" cycle sequencing kit (section 4.2.15.). For each set of recombinant plasmid clones, a stretch of 79 base pairs between primers MV3 and MV5 were analysed for changes from the consensus sequence (Figure 7.3.). The error rate of each amplification method was defined as the percentage of base changes from the consensus sequence for the total 790 bases analysed.

In a total of 790 bp sequenced, RT-nPCR generated no base changes and *rTth*-mediated RT-PCR generated 3 base changes (error rate = 0.38%).

For NASBA reaction products, cloned DNA which had been generated using a "Copy kit" possessed 3 base changes whilst that which had been generated by *rTth*-mediated RT-PCR contained 15 base changes (error rate = 1.9%). The difference in error rate between these two NASBA product cloning strategies was found to be statistically significant (p = 0.009) using a chi squared test. Table 7.1. summarises these results.

Table 7.1: Error rates for measles RNA amplification reactions

Reaction	RT-nPCR	<i>rTth</i> -mediated RT-PCR	NASBA followed by cDNA synthesis	NASBA followed by <i>rTth</i> - mediated RT-PCR
Error rate	0%	0.38%	0.38%	1.9%
Base changes		A→T C→T G→A	A→T G→C G→T	A→G (3) A→T C→G C→T G→C T→A (2) T→C (4) T→G (2)

Figure 7.3: Sequences of cloned measles RNA amplification products which contained base changes from the consensus sequence (Con)

DNA from r <i>Tth</i> -mediated RT-PCR	
Con	MV3- TGC CGA GGA TGC AAG GCT TGT TTC AGA GAT TGC AAT GCA
1.	MV3-T.....
Con	TAC TAC TGA GGA CAA GAT CAG TAG AGC AGT TGG ACC CAG A -MV5
1.T.....G..... -MV5
cDNA from NASBA reactions	
Con	MV3- TGC CGA GGA TGC AAG GCT TGT TTC AGA GAT TGC AAT GCA
4.	MV3-
10.	MV3-
Con	TAC TAC TGA GGA CAA GAT CAG TAG AGC AGT TGG ACC CAG A -MV5
4.T.....C..... -MV5
10.T..... -MV5
DNA from NASBA followed by r <i>Tth</i> -mediated RT-PCR	
Con	MV3- TGC CGA GGA TGC AAG GCT TGT TTC AGA GAT TGC AAT GCA
1.	MV3-C.....
2.	MV3-G.....C.....
3.	MV3-G.....
5.	MV3-C.....
6.	MV3-A.....C.....T.....
7.	MV3-G.....
8.	MV3-
9.	MV3-C.....
10.	MV3-
Con	TAC TAC TGA GGA CAA GAT CAG TAG AGC AGT TGG ACC CAG A -MV5
1. -MV5
2.G..... -MV5
3. -MV5
5.A..... -MV5
6. -MV5
7. -MV5
8.	G..... -MV5
9. -MV5
10.G.....T..... -MV5

7.2.3. Cloning and sequencing measles NASBA products generated from total intestinal RNA (Chapter 6).

For convenience, and the sake of cost, the second NASBA product cloning strategy was used for the analysis of NASBA products from samples 9 and 44 as described in the previous chapter. 10 µl of measles NASBA reactions were deproteinised and RNA recovered (section 4.2.8.). In addition, measles NASBA products from a positive control reaction (described in the previous chapter) containing Hu2 strain measles virus was processed in parallel in order to compare the measles sequences from samples 9 and 44 with the laboratory Hu2-strain virus.

1 ng of purified NASBA products was used as a template in an *rTth*-mediated RT-PCR reaction containing primers MV3 and MV5 (section 4.2.6.). Reaction products gel-purified and cloned into pT7Blue as described above. Ten recombinant plasmid clones from each sample were used for DNA sequencing as described above.

Following sequence analysis, the consensus sequences generated from samples 9, 44 and the Hu2 strain laboratory virus were all found to possess identical sequence homology.

7.3. DISCUSSION

Reaction products generated by RT-nPCR and *rTth*-mediated RT-PCR were successfully cloned into pT7Blue. Two strategies were employed to generate double stranded DNA from NASBA reaction products. The first strategy used a "Copy kit" to generate blunt-ended cDNA for cloning, while the second strategy used *rTth*-mediated RT-PCR to generate DNA for cloning.

In experiments to determine the relative fidelities of RT-nPCR and *rTth*-mediated RT-PCR, the results demonstrated that the former method had a higher fidelity than the latter method.


Experiments conducted to determine the relative fidelities of two strategies used to generate cloned NASBA reaction products showed that the first (generation of cDNA) was found to have a significantly higher fidelity than the second (*rTth*-mediated RT-PCR).


The fidelity of the first strategy was significantly lower than that reported by Sooknanan *et al.* (1994; error rates = 0.38% versus 0.03%) even though similar methods were used to amplify an RNA template generate double-stranded cDNA from NASBA products. Moreover, contrary to the results of Sooknanan *et al.* (1994), no deletions or insertions were observed in the sequences generated using this procedure. These differences may be explained by the use of different templates for amplification.

The low fidelity of the second strategy (error rate = 1.9%) is difficult to explain, especially since the individual fidelities of these reactions are relatively high (Table 7.1.). One explanation could be due to the fact that inosine triphosphate (ITP) is included in the NASBA reaction mix to improve the efficiency of the reaction. NASBA reaction products can therefore possess ITP. Inosine can base pair with all four bases (Martin *et al.* 1985) and it is possible that when NASBA products are ~~are~~ used as templates for *rTth*-mediated RT-PCR, the enzyme may incorporate any base when it reaches a position at which inosine is present in the template sequence. These "base changes" are detected following cloning and sequencing.

The large numbers of errors arising during NASBA followed by *rTth*-mediated RT-PCR shows that this approach to sequencing NASBA products can only be

employed when large numbers of cloned reaction products are available to generate an accurate consensus sequence. Conversely, double-stranded cDNA synthesis from NASBA RNA products generated fewer errors and therefore fewer plasmid clones are required to generate an accurate consensus sequence.

In experiments to determine the sequence of measles NASBA products derived from intestinal RNA samples 9 and 44 (described in the previous chapter). The results of the experiments involving samples 8 and 44 indicated that measles laboratory-strain positive control virus (Hu2; Taylor *et al.* 1991) was present. The measles Hu2-strain virus possesses a guanine to adenine substitution at 1336 of the viral genome. 

Eppendorf tubes in which NASBA reactions were carried out were not opened in the laboratory where reactions performed (University of Greenwich) and it therefore seems likely that contamination arose from positive control RNA from measles-infected Vero cells at the Royal Free Hospital. 

Chapter 8: EXPERIMENTS TO TEST HYBRID CAPTURE AND MULTIPLEX RT-PCR AS METHODS FOR MEASLES RNA DETECTION AND TO GENERATE A COMPETITIVE INTERNAL CONTROL

8.1 INTRODUCTION

Hybrid capture can increase the sensitivity of nucleic acid detection assays. In this project, two oligonucleotides (specific for measles genomic and noncoding N gene RNA) were adsorbed to polyvinylidene difluoride solid-phase supports and used to enrich total RNA samples for measles RNA prior to amplification reactions. oligonucleotide capture oligonucleotides were used in order to maximise the amount of measles RNA available for detection using this method.

To measure the efficiency of the hybrid capture and amplification, measles wild type RNA was generated from plasmid pT7BlueP as described in Chapter 5) were tested. In this study and PBMC homogenates used in these experiments prior to RNA isolation, hybrid capture and measles RNA amplification.

In this study, modified RNA transcripts were used as internal positive controls to measure the efficiency of RNA isolation and amplification, and as internal standards for quantification of measles RT-PCR in a manner analogous to that of Gilliland et al (1993). Such modified RNA transcripts give rise to RT-PCR products which can be distinguished from wild type RT-PCR products on the basis of size following agarose gel electrophoresis. In this study, a "spacer" DNA fragment was inserted into the MV1/MV2 measles insert possessed by the recombinant plasmid vector pT7BlueP (described in Chapter 5). Modified measles RNA transcripts were used as internal standards in later experiments for the detection of measles RNA (Chapter VI).

Multiplex PCR was developed in this study in order to develop a method capable of simultaneously detecting total RNA integrity and determining the presence of measles RNA. Multiplex RT-PCR involves the coamplification of two or more targets within the same reaction mixture (Edwards and Gibbs 1994). In this study, a multiplex RT-PCR reaction was developed which contained measles and U3A

Chapter 8: EXPERIMENTS TO TEST HYBRID CAPTURE AND MULTIPLEX RT-PCR AS METHODS FOR MEASLES RNA DETECTION AND TO GENERATE A COMPETITIVE INTERNAL CONTROL

8.1. INTRODUCTION

Hybrid capture can increase the sensitivity of nucleic acid detection methods. In this project, two oligonucleotides (specific for measles genomic and messenger N-gene RNA) were attached to paramagnetic solid-phase supports and used to enrich total RNA samples for measles RNA prior to amplification reactions. *Caf* oligonucleotide capture sequences were used in order to maximise the amount of measles RNA which could be enriched using this method.

To measure the efficiency of measles RNA isolation and amplification, measles wild type RNA transcripts (generated from plasmid pT7BlueE as described in Chapter 5) were "spiked" into tissue and PBMC homogenates used in these experiments prior to RNA isolation, hybrid capture and measles RNA amplification.

In this study, modified RNA transcripts were used as internal positive controls to measure the efficiency of RNA isolation and amplification, and as internal standards for quantitative, competitive RT-PCR in a manner analogous to that of Gilliland, *et al.* (1990). Such modified RNA transcripts give rise to RT-PCR products which can be differentiated from wild type RT-PCR products on the basis of size following agarose gel electrophoresis. In this study, a "spacer" DNA fragment was cloned into the MV1/MV2 measles insert possessed by the recombinant plasmid vector pT7BlueE (described in Chapter 5). Modified measles RNA transcripts were used as internal standards in later experiments for the detection of measles RNA (Chapter 9).

Multiplex PCR was also used in this study in order to develop a method capable of simultaneously assessing total RNA integrity and determining the presence of measles RNA. Multiplex RT-PCR involves the coamplification of two or more targets using two or more primer pairs (Edwards and Gibbs, 1994). In this study, a multiplex RT-PCR reaction was optimised which contained measles and U1A

primer pairs.

Aims. The aims of this part of the study were to:

1. Test whether hybrid capture could improve the sensitivity of measles RNA detection in intestinal tissue and PBMC samples.
2. Generate a modified measles RNA transcript for use as an internal positive control in measles RNA detection experiments.
3. Develop a measles RNA assay system using hybrid capture and internal positive control transcript RNA.
4. Develop a single multiplex RT-PCR method for the simultaneous determination of total RNA integrity using U1A and the presence of measles RNA.

8.2. RESULTS

8.2.1. Experiments to investigate the application of hybrid capture for measles RNA detection.

Efficiency of hybrid capture for measles RNA. Hybrid capture supports, derivatised with oligonucleotides HC1 and HC2 were synthesised as described in section 4.2.4. The ability of these supports to capture measles RNA was tested using measles wild type transcript RNA diluted in water (data not shown). Serial dilutions of such transcripts, from 10^6 to 1 molecules, were used for measles RNA hybrid capture (section 4.2.4.) together with *rTth*-mediated RT-PCR using primers MV3 and MV4 (section 4.2.6). Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; described in section 4.2.9.).

The detection sensitivity of measles RNA transcripts following the hybrid capture procedure was found to be equal to that without hybrid capture - i.e. 10 molecules. Therefore, this method of hybrid capture was capable of efficiently capturing measles RNA for use as a template in *rTth*-mediated RT-PCR reactions.

Hybrid capture enrichment of measles RNA (Figure 8.1.). A serial dilution of measles wild type RNA transcripts, from 10^7 to 10^3 molecules, was spiked into control intestinal resection tissue homogenates. Total RNA was isolated from these homogenates (section 4.2.3.), and all of the isolated RNA (up to 40 μ g) used for measles N-gene hybrid capture as described in section 4.2.4.

To compare the relative efficiencies of *rTth*-mediated RT-PCR and NASBA for the detection of measles RNA eluted from hybrid capture supports, eluted RNA was used for *rTth*-mediated RT-PCR with primers MV3 and MV4 (section 4.2.6; Figure 8.1.a. and b.), or NASBA using primers AB20 and AB22 (section 4.2.7; Figure 8.1.c. and d.). Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with radiolabelled probes (AB10 or U1A3; described in section 4.2.9.).

To measure any improvement in measles RNA detection sensitivity that measles hybrid capture conferred, an experiment was performed in which 10^6 to 10^2

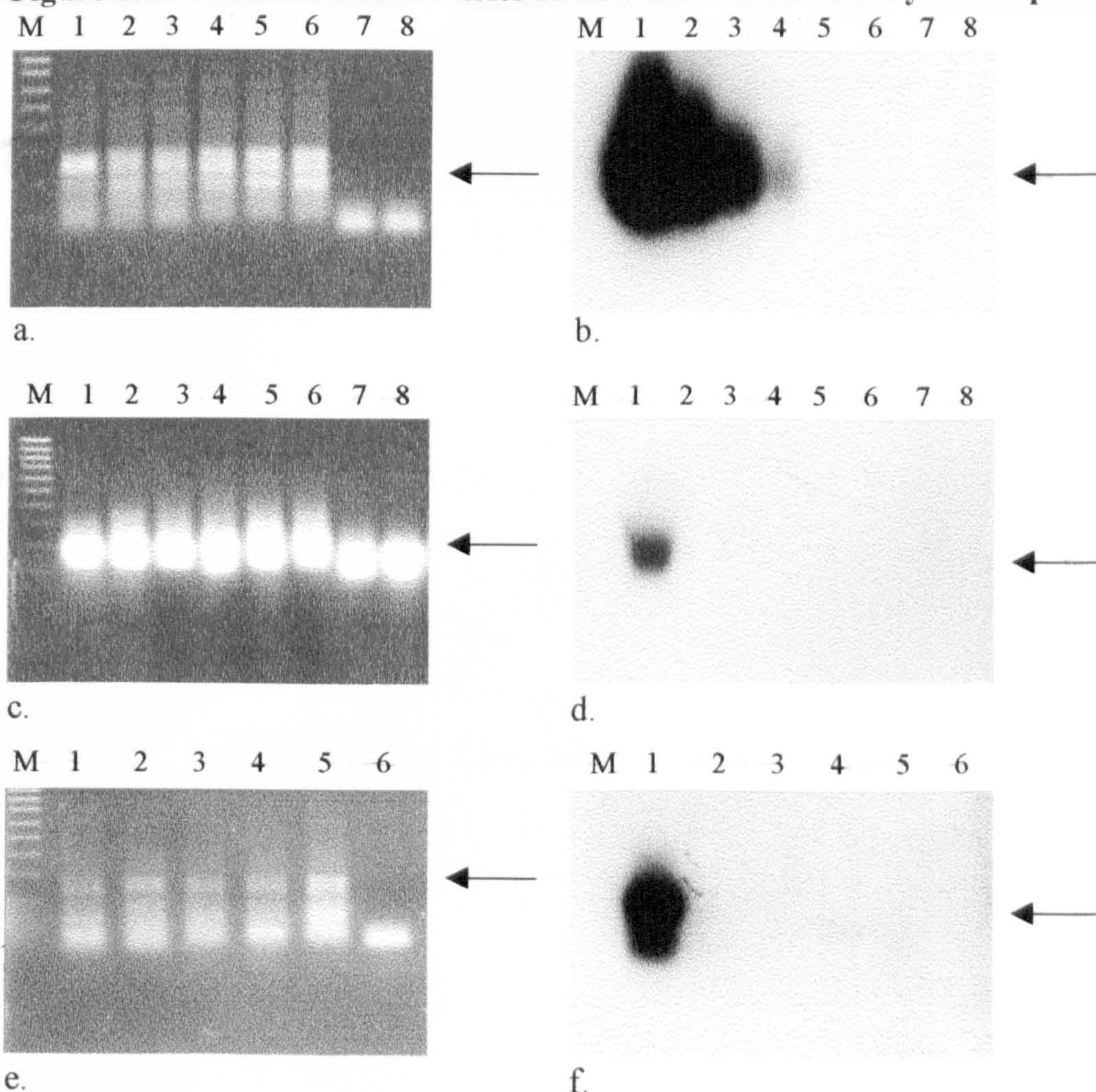
measles wild type RNA transcripts were "spiked" into control intestinal tissue homogenates. Total RNA was isolated from these homogenates (section 4.2.3.) and 100 ng of total RNA was subsequently used for *rTth*-mediated RT-PCR using primers MV3 and MV4 (Section 4.2.6; Figure 8.2.e. and f.). Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; section 4.2.9.).

It was observed that hybrid capture followed by *rTth*-mediated RT-PCR increased the ability to detect measles RNA 100-fold compared to *rTth*-mediated RT-PCR alone. This appeared as an improvement in the detection sensitivity limit from 10^6 to 10^4 measles transcript RNA molecules. This finding was also confirmed from results of experiments using total RNA from measles-infected Vero cells as a template for hybrid capture and *rTth*-mediated RT-PCR (data not shown).

Hybrid capture followed by NASBA was significantly less sensitive than hybrid capture followed by *rTth*-mediated RT-PCR for the detection of measles RNA; a detection sensitivity limit of 10^7 compared to 10^4 measles transcript RNA molecules "spiked" into control tissue homogenates.

All experiments were performed at least three times.

Figure 8.1: Detection of measles RNA with and without hybrid capture.



(a.) Gel and (b.) Southern blot analysis of measles *rTth*-mediated RT-PCR products. A serial dilution of wild type measles RNA transcripts (from 10^7 to 10^3 molecules; lanes 1 to 4) was spiked into intestinal tissue homogenates and hybrid capture performed on total isolated RNA (section 4.2.4.). Hybrid capture elutants were used for *rTth*-mediated RT-PCR of measles RNA (section 4.2.6.). Lanes 6 to 8 contain products from negative control reactions with water.

(c.) Gel and (d.) Northern blot analysis of measles NASBA reaction products (section 4.2.7.) using hybrid capture eluants from the above experiment. Lanes 6 to 8 contain products from negative control reactions with water.

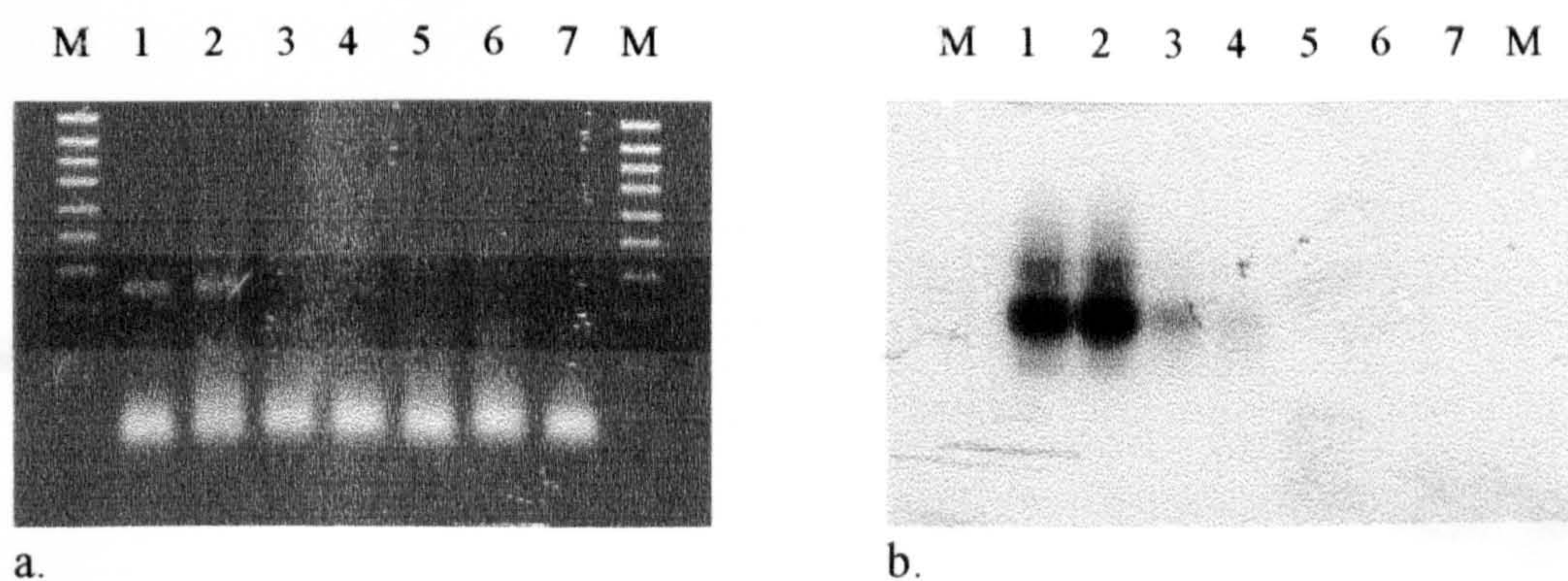
(e.) Gel and (f.) Southern blot analysis of measles *rTth*-mediated RT-PCR products. Total isolated RNA was isolated from intestinal tissue homogenates spiked with 10^6 to 10^2 measles wild type RNA transcripts (lanes 1 to 5). Lane 6 contains products from a negative control reaction with water.

All RT-PCR and NASBA products were probed with radiolabelled AB10 (section 4.2.9.). Lane M contains a 100 bp DNA marker.

Detection of measles RNA within total PBMC RNA (Figure 8.2). The sensitivity of measles RNA detection by *rTth*-mediated RT-PCR was determined by spiking a serial dilution from 10^7 to 10^2 wild type measles transcripts was spiked into control PBMC homogenates. Total RNA was isolated (section 4.2.3.) and 100 ng of total RNA used for *rTth*-mediated RT-PCR with primers MV3 and MV4 as described in section 4.2.6. Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; described in section 4.2.9.).

It was observed that as few as 10^4 wild type measles RNA transcripts could be detected in PBMC homogenates. This limit was not improved by applying hybrid capture (data not shown).

Figure 8.2: Sensitivity of measles N-gene RNA detection in PBMCs.



(a.) Gel and (b.) Southern blot analysis of *rTth*-mediated RT-PCR products (section 4.2.6.), using total RNA from PBMC homogenates spiked 10^7 to 10^2 measles wild type transcripts (lanes 1 to 6). Lane 7 contains products from a negative control reaction with water.

RT-PCR products were probed with radiolabelled AB10 (section 4.2.9.). Lane M contains a 100 bp DNA size marker.

8.2.2. Generation of an internal positive control RNA transcript.

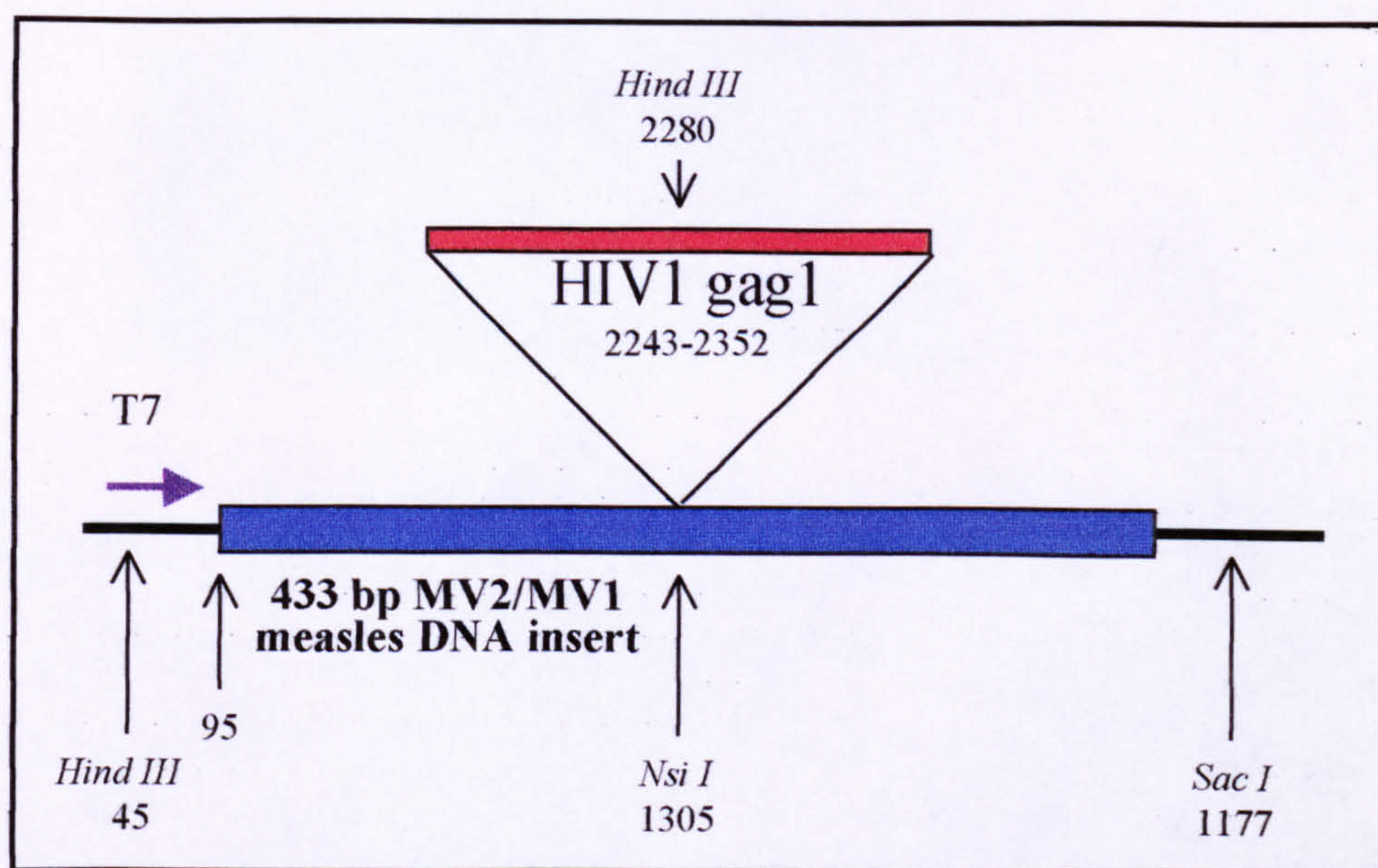
Modification of pT7BlueE (Figure 8.3.). A "spacer" DNA fragment was cloned into pT7BlueE at the *NsiI* restriction site possessed by the MV1/MV2 DNA insert. Briefly, 10 ng of a 144 bp *PstI* / *SphI* restriction fragment (Table 4.2.) was blunt ended using T4 DNA polymerase (section 4.2.11.). 5' DNA termini of the DNA recovered from this reaction were phosphorylated using T4 polynucleotide kinase (section 4.2.11.).

1 µg of pT7BlueE was digested with *NsiI* (section 4.2.12.) the ends of the linearised plasmid blunt ended with T4 DNA polymerase (section 4.2.12.) and 5' termini dephosphorylated using alkaline phosphatase (section 4.2.9.).

Insert and plasmid DNA were ligated using T4 DNA ligase (section 4.2.10) and 1µl of ligation products transformed into competent *E.coli* (section 4.2.11.). 12 transformed bacterial colonies were cultured and plasmid DNA isolated (section 4.2.13.). Purified plasmids were screened for the presence of HIV1 gag1 insert DNA by digestion with *HindIII* (section 4.2.13.) since a *HindIII* restriction site is unique to the HIV1 gag1 insert DNA sequence (Figure 8.3.).

The modified plasmid generated from this experiment - termed "pT7BlueE144" - was observed to contain an insert DNA sequence which was 130 bp, rather than 144 bp, larger than the original MV1/MV2 insert DNA sequence. This result is due to the fact that a total of 14 bp of DNA were removed from insert and vector sequences by T4 DNA polymerase during the blunt-ending procedures.

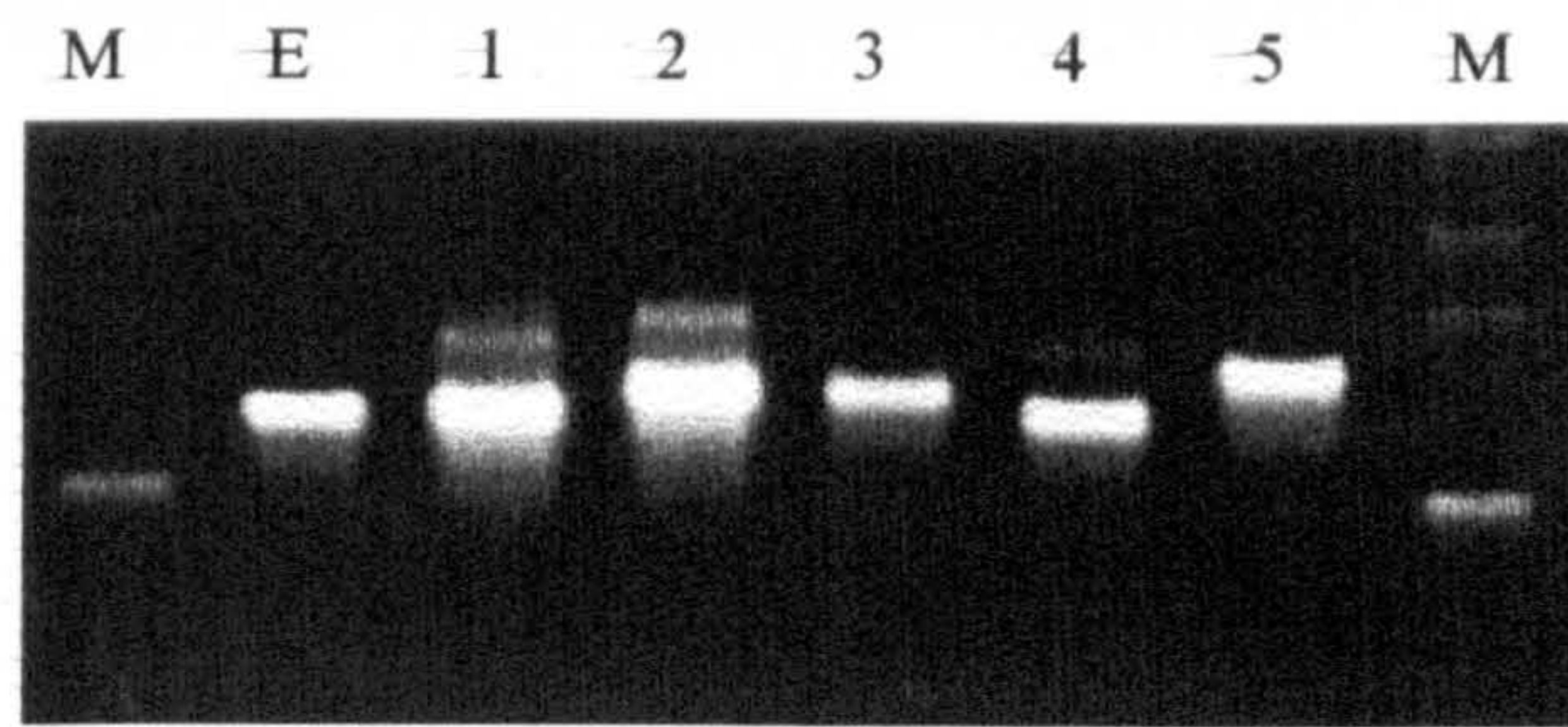
Figure 8.3: Diagram of pT7BlueE144 containing the HIV1 gag1 DNA insert.



In vitro transcription (Figure 8.4.). pT7BlueE144 was linearised downstream from the insert DNA sequence by digestion with *EcoRI* (section 4.2.17.) and 2 µg of linearised plasmid recovered from this reaction was used as a template for *in vitro* transcription (4.2.17.).

Following *in vitro* transcription, plasmid DNA was removed by digestion with DNaseI and modified measles RNA transcripts (636 nt in length) of negative sense were recovered from the mixture (section 4.2.14.). 1 µg of transcript RNA was analysed by agarose gel electrophoresis (section 4.2.8.), its concentration determined spectrophotometrically (section 4.2.8.), and this concentration converted from ng µl⁻¹ to numbers of molecules µl⁻¹ as the length of the RNA transcript was known. A stock serial dilution of transcript RNA was made up in water, dispensed into aliquots containing a total of 10¹⁰ molecules to 1 molecule, and stored at -70°C until required.

Figure 8.4: Gel analysis of modified measles transcript RNA.



RNA gel analysis of modified measles RNA transcript generated by *in vitro* transcription of pT7BlueE144. Lane E contains transcripts from pT7BlueE with a predicted size of 506 nt, and lane 2 contains transcripts from pT7BlueE144 with a predicted size of 636 nt. Lane M contains an RNA size marker (0.24 to 9.5 nt; Table 4.3.).

8.2.3. Development of a measles RNA assay system.

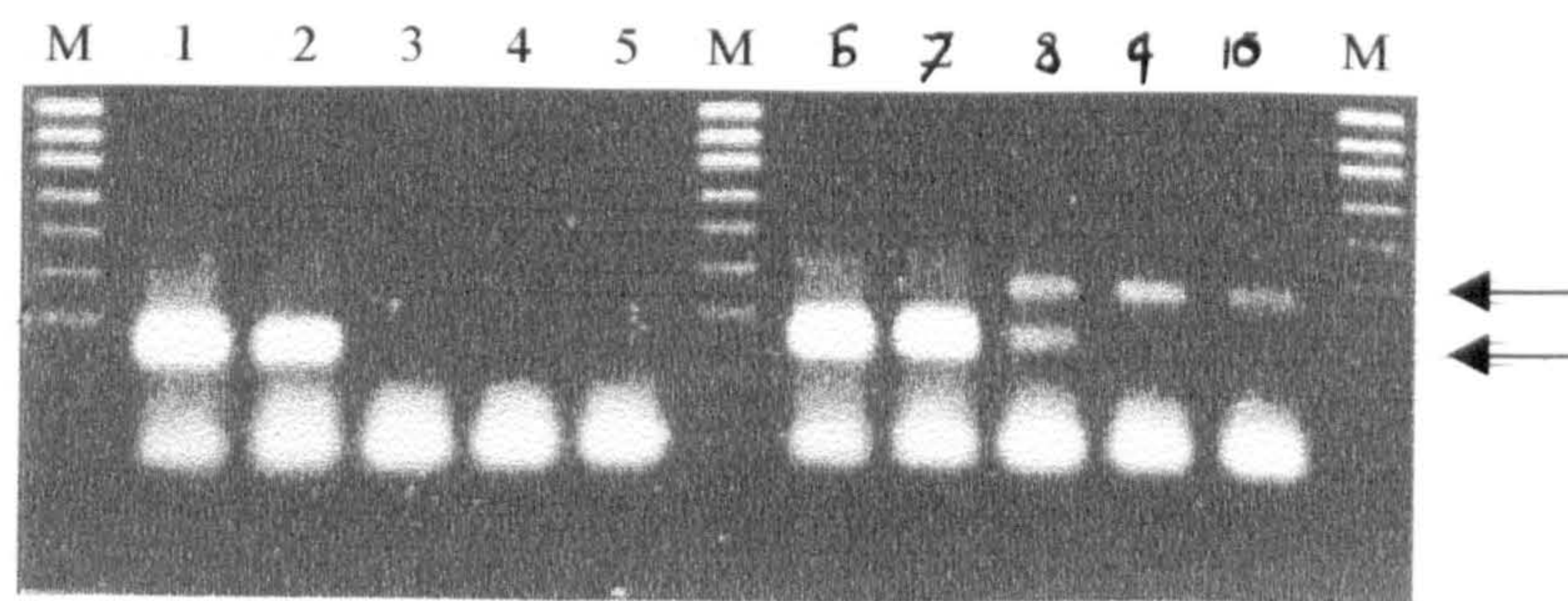
Hybrid capture of modified measles RNA transcripts (data not shown). A serial dilution of modified measles RNA transcripts, from 10^8 to 10 molecules, was spiked into control intestinal resection tissue homogenates. Total RNA isolation (4.2.3.), hybrid capture (section 4.2.4.) and *rTth*-mediated RT-PCR (section 4.2.6.) was performed and reaction products analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; described in section 4.2.9.).

It was observed that 10^6 modified measles RNA transcripts could be detected.

Coamplification of wild type and modified measles transcript RNAs (Figure 8.5.). A serial dilution of wild type measles RNA transcripts, from 10^3 to 1 transcript molecules, was amplified by *rTth*-mediated RT-PCR using primers MV3 and MV4 (section 4.2.6.). In a second set of reactions, 10^3 modified measles RNA transcripts were added to a similar dilution series of wild-type measles RNA transcripts (from 10^3 to 1 transcript molecules) and amplification carried out in the same manner. Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; described in section 4.2.9.).

The ability to detect measles RNA was observed to be unimpaired when the modified measles RNA transcripts were present together with the wild type measles RNA transcripts.

Figure 8.5: Competitive RT-PCR using wild type and modified measles RNA transcripts.



Gel analysis of *rTth*-mediated RT-PCR products using a serial dilution of measles wild-type transcript without (lanes 1 to 5) or with (lanes 6 to 10) 10^3 molecules of modified measles transcript. Lanes 1 to 5 and 6 to 10 contained products of reactions with 10^3 , 10^2 , 10 , 1 and 0 wild-type transcript molecules.

The upper arrow points to 383 bp modified transcript RT-PCR products and the lower arrow points to 253 wild type transcript RT-PCR products. Lane M contains a 100 bp DNA marker (Table 4.3.).

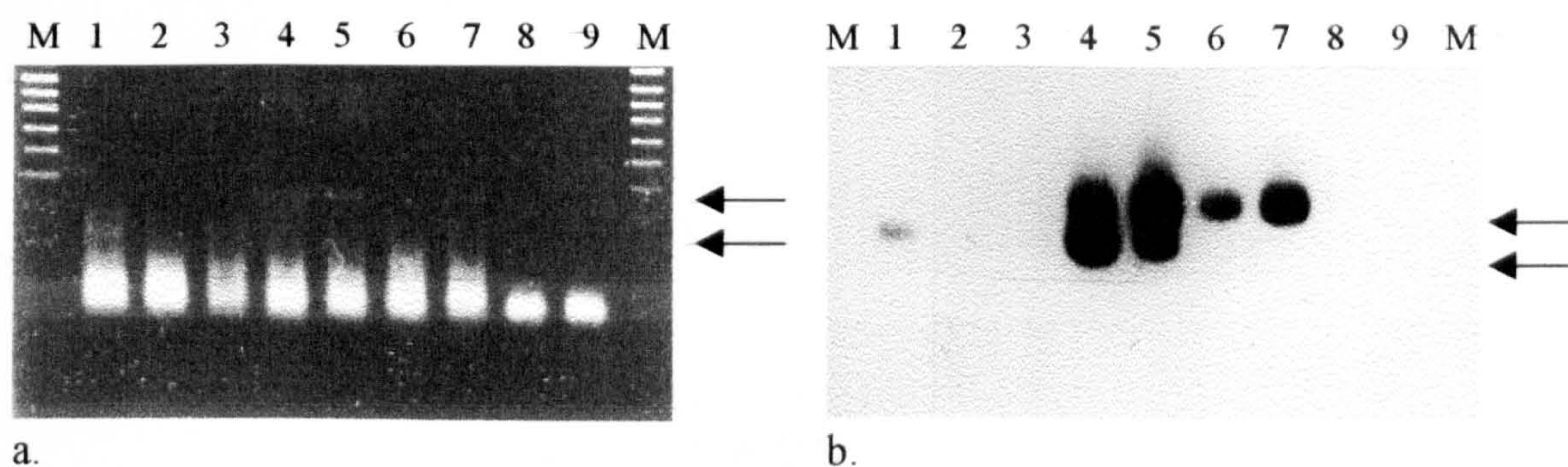
Detection of spiked wild type and modified measles transcript RNAs using hybrid capture followed by RT-PCR (Figure 8.6.). 10^5 , 10^4 , and 10^3 wild type measles RNA transcripts were spiked into one set of tissue homogenates. Another set of tissue homogenates was spiked with 10^5 , 10^4 , and 10^3 wild type measles RNA transcripts together with 10^7 modified measles transcripts. 10^7 modified measles RNA transcripts were used as this number represented the range of the limit of detection sensitivity as described previously.

Total RNA was isolated (section 4.2.3.), hybrid capture performed (section 4.2.4.) and measles transcript RNAs detected by *rTth*-mediated RT-PCR using primers MV3 and MV4 (section 4.2.6.). Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; section 4.2.9.).

It was observed that the presence of modified measles transcript RNA did not diminish the efficiency of wild type measles transcript isolation. Hybrid capture and amplification permitted the detection of as few as 10^4 wild type measles RNA transcripts as well as 10^7 modified measles RNA transcripts.

This experiment was repeated at least three times to ensure reproducibility of results.

Figure 8.6: Detection sensitivity of hybrid capture and competitive RT-PCR for measles RNA.



(a.) Gel and (b.) Southern blot analysis of measles *rTth*-mediated RT-PCR products. Intestinal tissue homogenates 1 to 3 (lanes 1 to 3) were spiked with 10^5 , 10^4 , and 10^3 measles wild type RNA transcripts. Intestinal tissue homogenates 4 to 7 (lanes 4 to 7) were spiked with 10^5 , 10^4 , and 10^3 measles wild type RNA transcripts together with 10^7 modified measles RNA transcripts. Lanes 8 and 9 contain products from negative control reactions with water. RT-PCR products were probed with radiolabelled AB10 (section 4.2.9.). Lane M contains a 100 bp DNA size marker (Table 4.3.).

8.2.4. Development of a multiplex *rTth*-mediated RT-PCR assay.

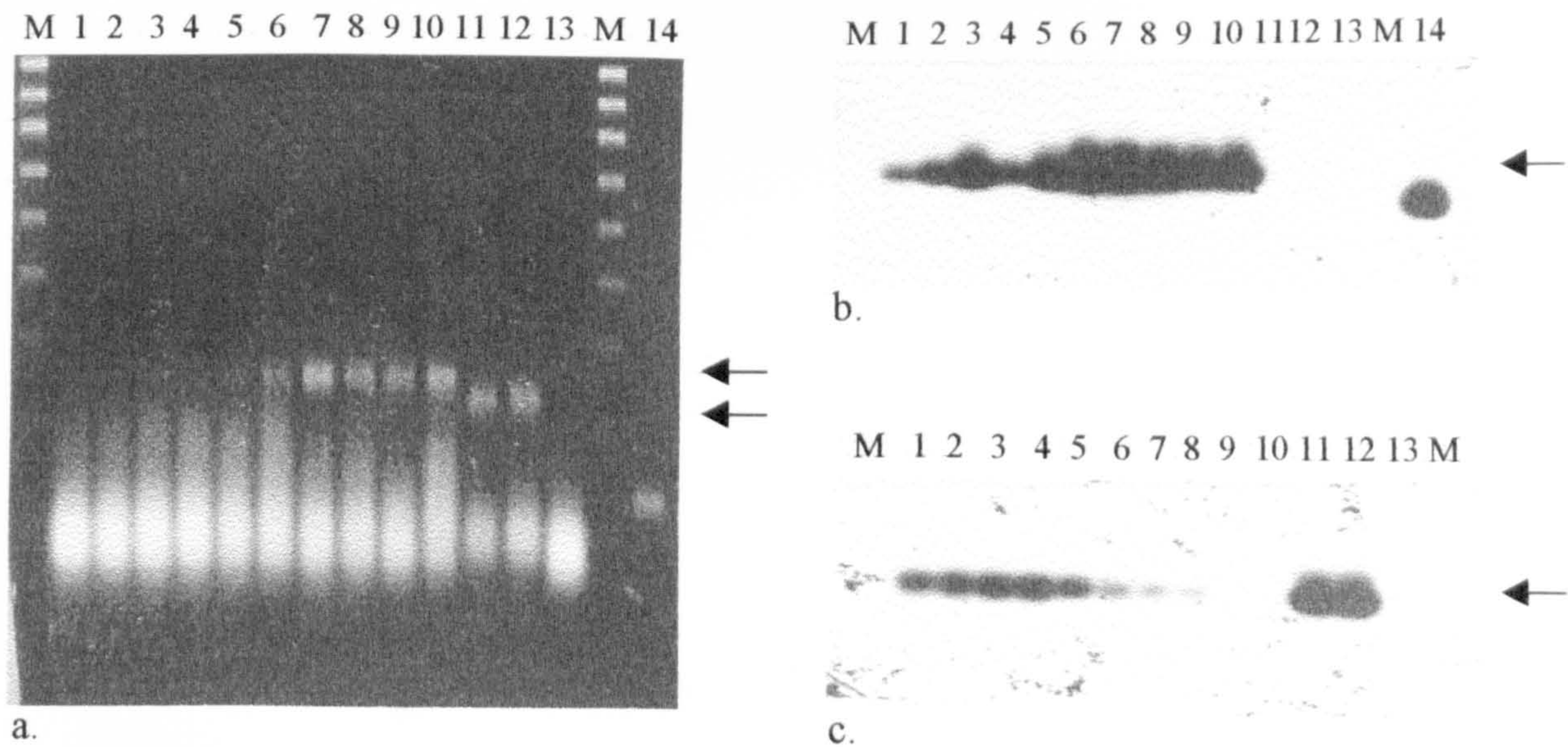
Optimisation of U1A and measles N-gene coamplification. 10 ng of total RNA isolated from intestinal resection tissue (section 4.2.3.) was spiked with 10⁴ wild type measles RNA transcripts and the effect of different concentrations of U1A and measles primers on the efficiency of *rTth*-mediated RT-PCR was determined. Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with radiolabelled probes (AB10 and U1A3; described in section 4.2.9.) to differentiate between U1A and measles-specific PCR products. The different concentrations of U1A and measles primer pairs used in these experiments are shown in Table 8.1.

From these experiments, it was observed that 750 nM primers MV3 and MV4, and 150 nM primers U1A1 and U1A2 was the optimal primer combination for multiplex RT-PCR of both U1A and measles RNA (Figure 8.6.).

Table 8.1: Measles and U1A primer concentrations for multiplex RT-PCR

Reactions	Total MV3/MV4 conc. (nM)	Total U1A1/U1A2 conc. (nM)
1,2	450	450
3,4	550	350
5,6	650	250
7,8	750	150
9,10	900	0
11,12	0	900
13	450	450

Figure 8.7: Multiplex *rTth*-mediated RT-PCR for measles and U1A RNA.



(a.) Gel analysis of *rTth*-mediated multiplex RT-PCR products (section 4.2.6.). U1A and measles primer concentrations are given in Table 8.1. Lanes 1 to 12 contain products from reactions with 10 ng total RNA from human intestinal tissue and 10^4 wild type measles RNA transcripts. Lane 13 contains products from a negative control reaction with water. Lane 14 contains an MV3/MV5 PCR product as a positive control for probe hybridisation.

(b.) Southern blot analysis of the gel from (a) probed with radiolabelled AB10 (section 4.2.9.).

(c.) Southern blot analysis of the gel from (a) probed with radiolabelled U1A3 (section 4.2.9.).

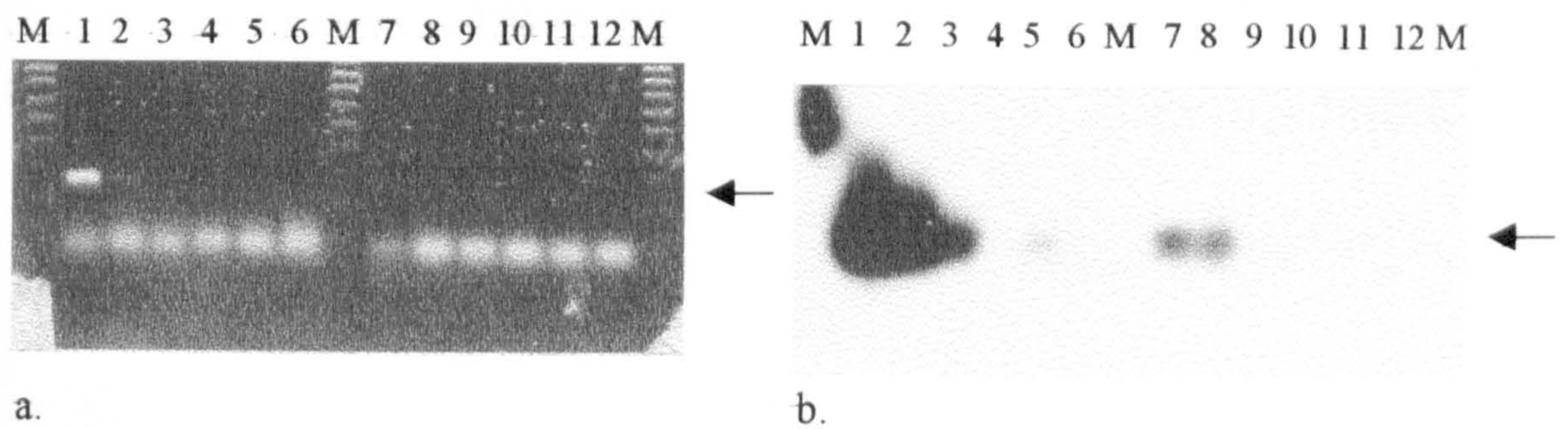
Lanes M contain a 100 bp DNA marker (Table 4.3.).

Multiplex *rTth*-mediated RT-PCR for U1A RNA, measles wild type RNA transcripts and modified measles RNA transcripts. The optimised multiplex *rTth*-mediated RT-PCR assay described above was further developed to address the amplification of three RNA templates using 10 ng total intestinal RNA, 10^3 modified measles RNA transcripts, and a serial dilution of measles wild type

transcripts, from 10^5 to 10 wild type measles RNA transcripts. Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; described in section 4.2.9.).

It was observed that the addition of modified measles RNA transcripts to the previous reaction diminished the apparent sensitivity of measles wild type transcript detection by a factor of ten (Figure 8.7.).

Figure 8.8: Multiplex RT-PCR for U1A RNA, wild type and modified measles transcript RNAs.



(a.) Gel and (b.) Southern blot analysis of *rTth*-mediated multiplex RT-PCR products (section 4.2.6.). Lanes 1 to 5 contain products from reactions with primers MV3 and MV4 with 10^5 to 10 measles wild-type transcripts. Lanes 7 to 11 contain products of reactions with primers MV3, MV4, U1A1 and U1A2 with 10^5 to 10 measles wild-type transcripts, 10 ng of total intestinal RNA and 10^3 modified measles transcripts. Lanes 6 to 12 contain products from negative control reactions with water.

RT-PCR products were probed with radiolabelled AB10 (section 4.2.9.).

Lane M contains a 100 bp DNA size marker.

8.4. DISCUSSION

The results presented here indicate that hybrid capture increases the detection sensitivity of measles RNA within total RNA from tissue homogenates by a factor of 100 permitting the detection of as few as 10^4 measles RNA transcripts and represents the first time that magarose solid phase supports have been applied to the enrichment of RNA species.

However, hybrid capture did not improve the detection sensitivity of measles RNA in PBMC homogenates.

Hybrid capture and *rTth*-mediated RT-PCR was observed to be more sensitive than hybrid capture and NASBA (Figure 8.1.). This was unexpected as previously NASBA was found to be more sensitive than *rTth*-mediated RT-PCR for the detection of measles RNA in water (Chapter 5). This observation could have been due to a susceptibility of NASBA to substances carried over from the hybrid capture procedure. Such poor robustness and versatility limit the use of NASBA to the detection of measles in RNA samples diluted in water.

A modified measles RNA transcript was successfully produced by cloning a "spacer" DNA fragment into the MV1/MV2 sequence of pT7BlueE. These transcripts were used as an internal positive control in later experiments (Chapter 9). This transcript did not interfere with the detection of wild type measles RNA.

It was observed that hybrid capture followed by RT-PCR could detect fewer wild type transcripts (10^4) than modified transcripts (10^6). This can be explained by the inefficient binding of modified transcripts to the hybrid capture sequences as a result of insertion of HIV1 gag1 DNA at the *NsiI* site interrupting the capture sequence target site.

Previously, Albretsen *et al.* (1990) have used a hybrid capture method for the detection of measles RNA. Their method was not used in this study as it was thought more appropriate to use measles N- rather than measles H-gene RNA as a target for detection. The characteristic 5' to 3' measles RNA transcription gradient leads to a greater abundance of measles N-gene RNA transcripts compared to other measles RNA transcripts in infected cells.

Also, the method developed by Albretsen *et al.* only captured messenger and

antigenomic measles (sense) RNA, but in infected cells, measles genomic (antisense) RNA is also present. A strategy which only permitted the capture and detection of measles sense RNA is likely to be less sensitive than one which detected both senses together.

Finally, this method developed could in theory form the basis of a general way by which other viral RNA species, such as mumps or rubella.

✓ measles
rubella.

Chapter 9

Chapter 9: EXPERIMENTS TO DETECT MEASLES RNA IN IBD INTESTINAL AND PBMC CLINICAL SAMPLES USING HYBRID CAPTURE FOLLOWED BY RT-PCR.

9.1. INTRODUCTION

The combined measles N-gene-specific hybrid capture and *rTth*-mediated RT-PCR method was used to enrich and amplify measles RNA present in total cellular RNA isolated from IBD and control intestinal tissue samples. Samples were: Crohn's disease tissue (n=10), ulcerative colitis tissue (n=4) and tissue from control patients undergoing resection for intestinal carcinoma (n=6) (Table 9.1.). All IBD tissues showed evidence of inflammation on histological examination (data not shown). All Crohn's disease tissues were selected on the basis that they contained granulomas.

In addition to intestinal resection tissue, PBMC samples from IBD and control patients were also analysed for the presence of measles RNA. Blood samples for PBMC isolation were taken from patients with Crohn's disease (n=6), indeterminate colitis (n=3) ulcerative colitis (n=2) and control patients (n=2). Blood samples were also obtained from two patients with SSPE. Verbal consent was obtained from all IBD and control patients providing blood samples for this study. Ethics approval was obtained from the Royal Free Hospital Ethics Committee. Patient details are given in Table 9.1.

RT-nPCR for measles H-gene RNA, using a previously published method (Kawashima *et al.* 1996b), was performed in order to confirm measles N-gene RT-PCR.

RNA isolation, preparation of RT-PCR reaction mixtures and analysis of RT-PCR products were performed in separate laboratories, this time at the Royal Free Hospital, in order to minimise the possibility of PCR contamination.

Table 9.1: Details of patients used for measles RNA analysis.

Patient	Disease	Age (yrs)	Sex (M/F)
Intestinal tissue			
1	Crohn's	25	M
2	UC	51	M
3	Sigmoid volulus	60	M
4	Crohn's	22	F
5	Crohn's	36	F
6	Crohn's	17	F
7	UC	55	M
8	Crohn's	41	M
9	UC	52	F
10	UC	58	M
11	Ischaemia	49	F
12	Crohn's	36	F
13	Carcinoma	78	F
14	Carcinoma	68	F
15	Crohn's	40	M
16	Crohn's	27	M
17	Carcinoma	52	M
18	Carcinoma	45	F
19	Crohn's	21	F
20	Crohn's	37	F
SSPE tissue			
SSPE1	SSPE	21	M
SSPE2	SSPE	11	M
SSPE3	SSPE	14	M
SSPE4	SSPE	6	M
Peripheral blood cells			
A	Indeterminate colitis	45	F
B	UC	74	F
C	Crohn's	50	M
D	Crohn's	25	F
E	Crohn's	55	F
F	Crohn's	34	M
G	Indeterminate colitis	41	F
H	Crohn's	49	M
I	Indeterminate colitis	13	F
J	Rectal bleed	8	M
K	UC	14	M
L	Crohn's	16	M
M	Autoimmune hepatitis	32	F
Positive control PBMCs			
X	Vaccine recipient	NA	NA
Y	SSPE	NA	M
Z	SSPE	NA	M

Aims. The aims of this study were to:

1. Isolate total RNA from a coded series of resection tissue specimens and blood samples from IBD and control patients, and assess its integrity.
2. To analyse IBD and control RNA samples for the presence of measles N-gene RNA using hybrid capture followed by *rTth*-mediated RT-PCR and to analyse the same samples for the presence of measles H-gene RNA using RT-nPCR.
3. To characterize the strain of measles virus present by sequence analysis of measles RT-PCR products.

h^a

9.2. RESULTS

9.2.1. Total RNA isolation and integrity.

Tissue samples (Figure 9.1.). Coded fresh-frozen intestinal resection tissue samples were obtained as described in section 4.2.3. Intestinal tissue homogenates were divided into two equal portions and 10^7 modified measles RNA transcripts added as an internal positive control to assess any negative effect on measles RNA detection that the isolation procedure may have had. Total RNA isolations were performed in duplicate (section 4.3.2.).

As a positive control to assess the methods/~~general~~ ability to detect measles RNA within persistently infected tissue, RNA was isolated from four SSPE autopsy brain samples as described in section 4.2.3. Patient details are given in Table 9.1.

To assess the integrity of total RNA isolated from intestinal ~~of~~ SSPE tissue samples, 100 ng of RNA was used for *rTth*-mediated RT-PCR for U1A RNA (section 4.2.6.) using primers U1A1 and U1A2. Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (U1A3; section 4.2.9.). In addition, total RNA was analysed by agarose gel electrophoresis (section 4.2.8.).

All four SSPE RNA samples, and all but two intestinal RNA samples yielded the expected amplification products following *rTth*-mediated RT-PCR for U1A RNA. 28S and 18S ribosomal RNA bands were clearly visible in all RNA samples following agarose gel electrophoresis.

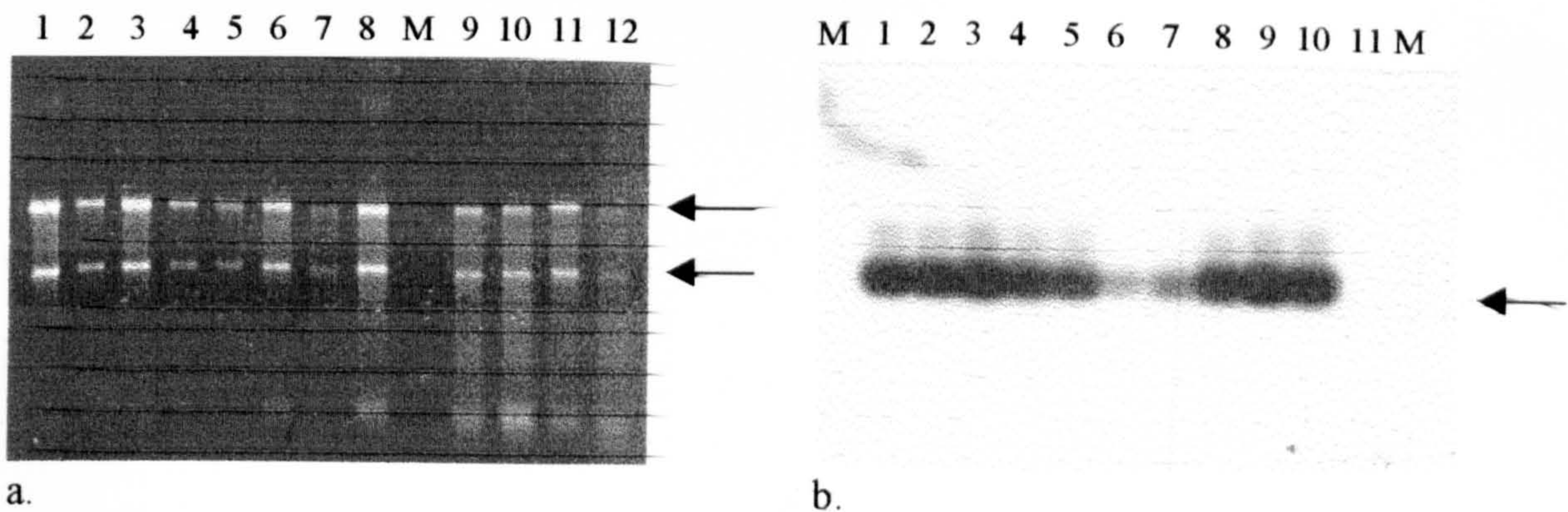
PBMC samples (Figure 9.1.). PBMCs were prepared from whole blood, and total RNA isolated (4.2.3.). 5 µl of PBMC RNA samples were used for agarose gel analysis (section 4.2.8.) and *rTth*-mediated RT-PCR for U1A RNA (section 4.2.6.) using primers U1A1 and U1A2. Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (U1A3; section 4.2.9.).

As a positive control, PBMC RNA from a measles vaccine (strain AIK-C) recipient was supplied by H. Kawashima (Tokyo Medical School, Japan).

All but one PBMC sample yielded the expected amplification products

following *rTth*-mediated RT-PCR for U1A RNA and 28S and 18S ribosomal RNA bands were clearly visible in all RNA samples following agarose gel electrophoresis.

Figure 9.1: Analysis of isolated total intestinal RNA integrity.



(a.) Representative gel analysis (section 4.2.8.) of 5 μ l of total RNA from PBMC samples A to H (lanes 1 to 8), and 1 μ g total RNA from SSPE autopsy material (lanes 9 to 12). The upper and lower arrows point to 28S and 18S ribosomal RNA band, respectively. Lane M contains an RNA size marker (0.24-9.5 nt; described in Table 4.3.)

(b.) Representative Southern blot analysis *rTth*-mediated RT-PCR products from reactions containing intestinal RNA (lanes 1a to 5b). Lane 11 contains products from a negative control reaction with water. PCR products were probed using radiolabelled U1A3 (section 4.2.9.). The arrow points to a 225 bp PCR product. Lane M contains a 100 bp DNA size marker (Table 4.3.).

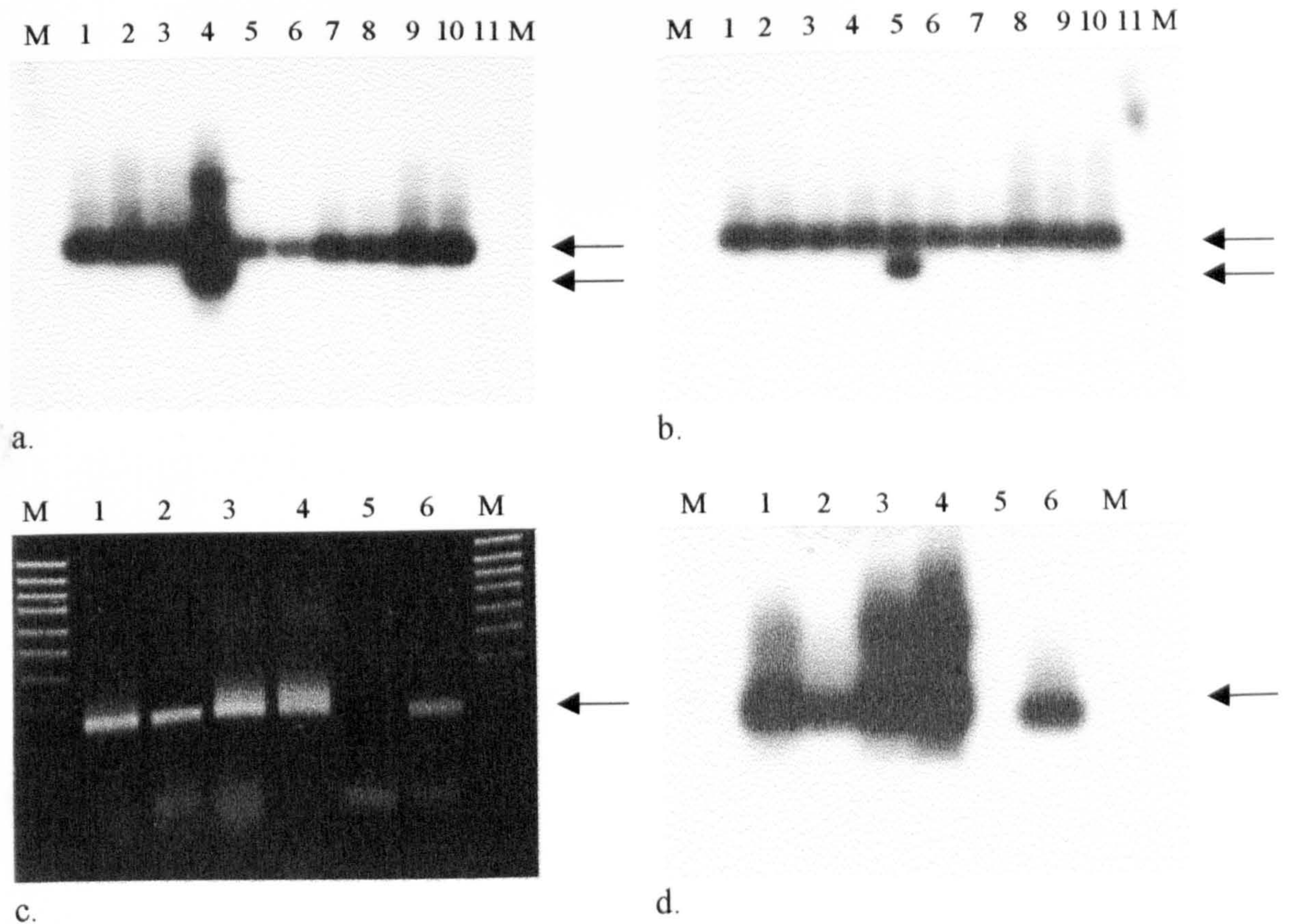
9.2.2. Detection of measles RNA.

Detection of measles N-gene RNA in tissue samples (Figure 9.2.). Hybrid capture and *rTth*-mediated RT-PCR for measles N-gene RNA was performed on duplicate intestinal RNA samples as previously described. As a positive control, *rTth*-mediated RT-PCR for measles N-gene RNA was performed on 100 ng of SSPE RNA (section 4.2.6.). Reaction products were analysed by agarose gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10; described in section 4.2.9.)

Internal modified measles RNA transcripts were successfully amplified from all isolated intestinal RNA samples. Two samples (3a and 11b) yielded wild type RT-PCR products and these samples were derived from one patient with Crohn's disease (patient 3) and one non-IBD control patient (patient 11).

All four SSPE samples yielded positive results by *rTth*-mediated RT-PCR for measles N-gene RNA.

Figure 9.2: Analysis of intestinal and SSPE tissue for measles N-gene RNA.



Southern blot analysis of *rTth*-mediated RT-PCR products (section 4.2.6.) from measles N-gene hybrid capture experiments using intestinal RNA samples 1a-5a (a.) and 10a-15a (b.). Lane 11 contains products from a negative control reactions with water. The upper arrows point to a 383 bp RT-PCR product resulting from the amplification of the modified measles RNA transcript, and the lower arrows point to a 253 bp RT-PCR product resulting from the amplification of wild type measles RNA.

(c.) Gel and (d.) Southern blot analysis of measles N-gene *rTth*-mediated RT-PCR products using total RNA from SSPE autopsy samples 1 to 4 (lanes 1 to 4). All RT-PCR products were probed with radiolabelled AB10 (section 4.2.9.). Lane M contains a 100 bp DNA size marker.

Detection of measles H-gene RNA in tissue samples (data not shown). Measles H-gene RT-nPCR (section 4.2.5.) was performed using 1 µg of intestinal or SSPE RNA for RT with primer MF1, followed by nested PCR reaction with primer pairs H3 and H6 (first round), then H4 and H7 (second round). Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (H5; described in section 4.2.9.).

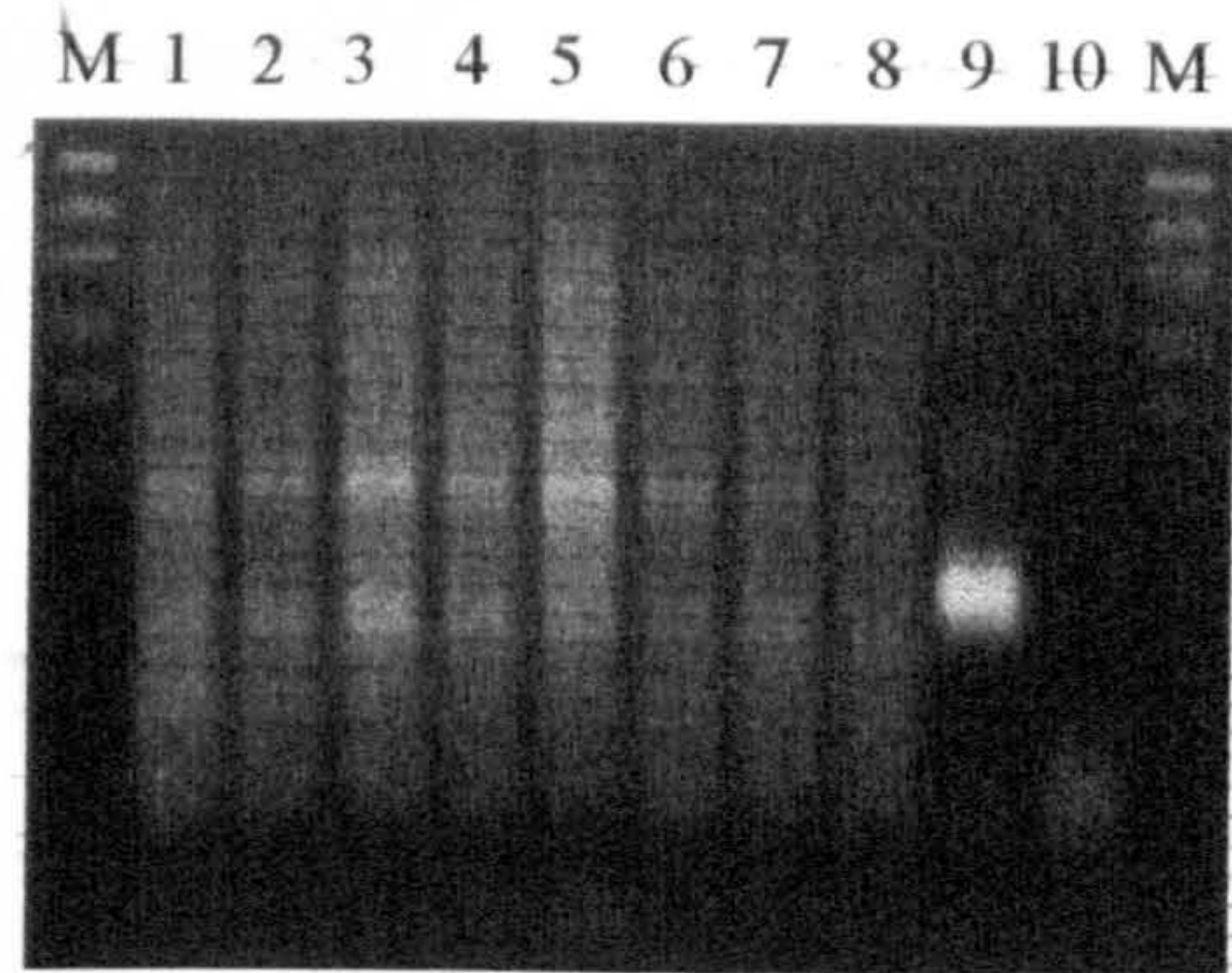
No intestinal RNA samples were observed to be positive for measles H-gene RNA although all four SSPE samples yielded positive results.

Detection of measles RNA in PBMC samples (Figure 9.3.). 5 µl of RNA from PBMC samples were used for *rTth*-mediated RT-PCR for measles N-gene RNA (section 4.2.6.). Additionally, 5 µl of RNA from PBMC samples were used for RT-nPCR for measles H-gene RNA (section 4.2.5.). Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (AB10 or H5; described in section 4.2.9.) to achieve maximum sensitivity.

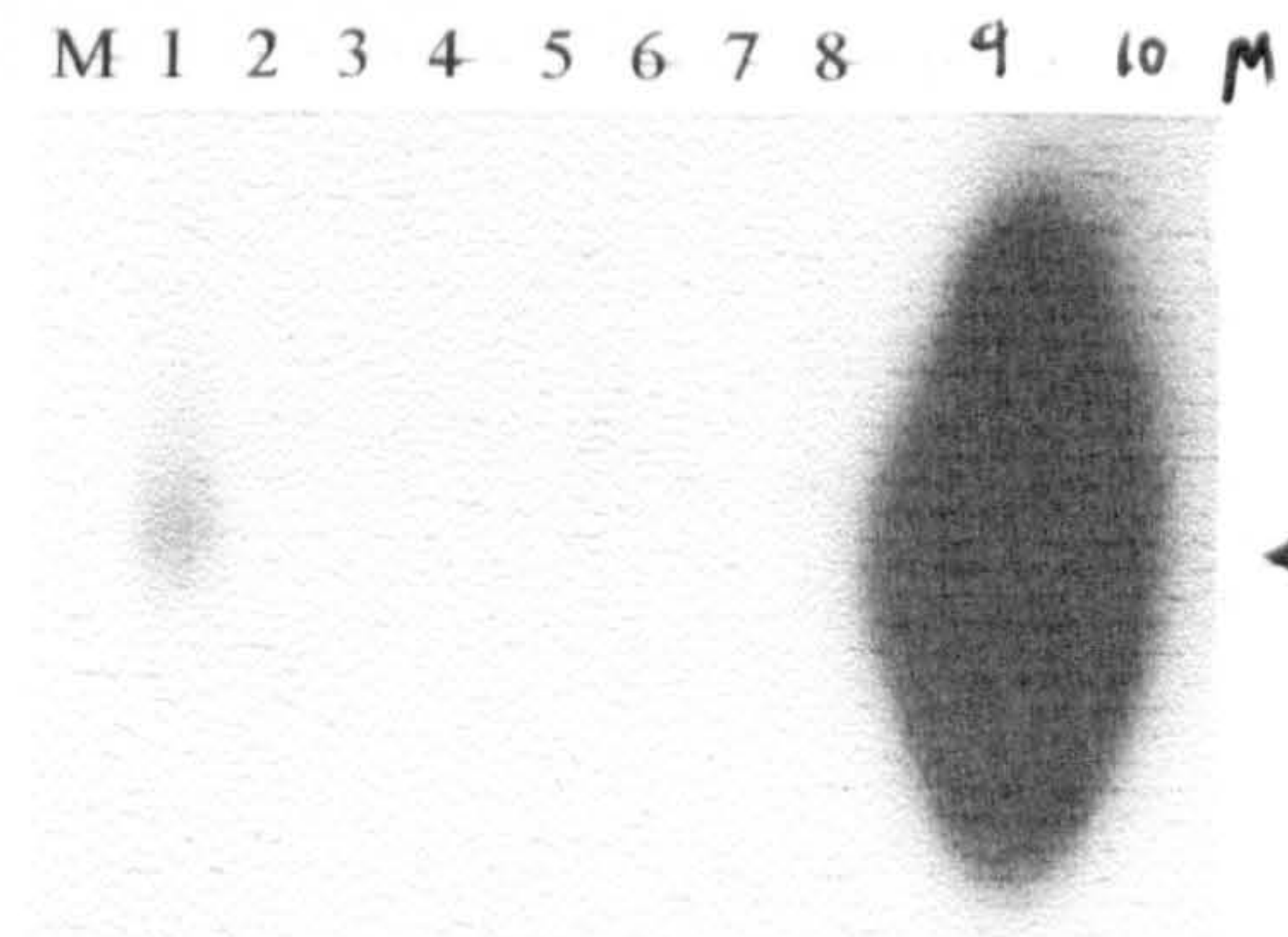
One PBMC sample, derived from a patient with indeterminate colitis (patient A), was found to be positive for measles N-gene RNA. All other PBMC RNA samples were found to be negative for measles N-gene RNA, including PBMC RNA from two patients with SSPE (data not shown).

The measles vaccine recipient PBMC RNA sample was found to be positive for measles H-gene RNA. All other PBMC RNA samples were observed to be negative for measles H-gene RNA.

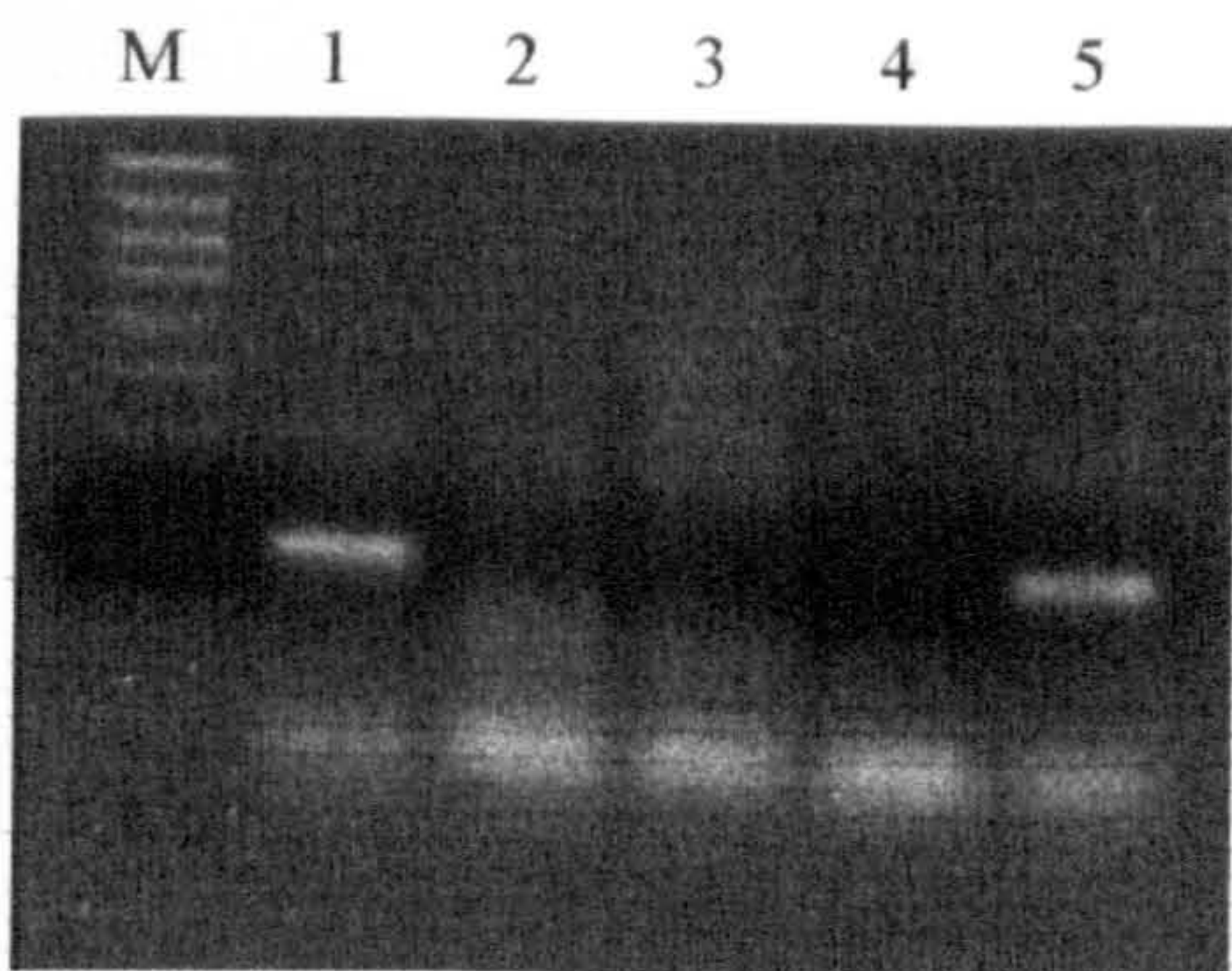
Figure 9.3: Analysis of PBMC samples for measles RNA.



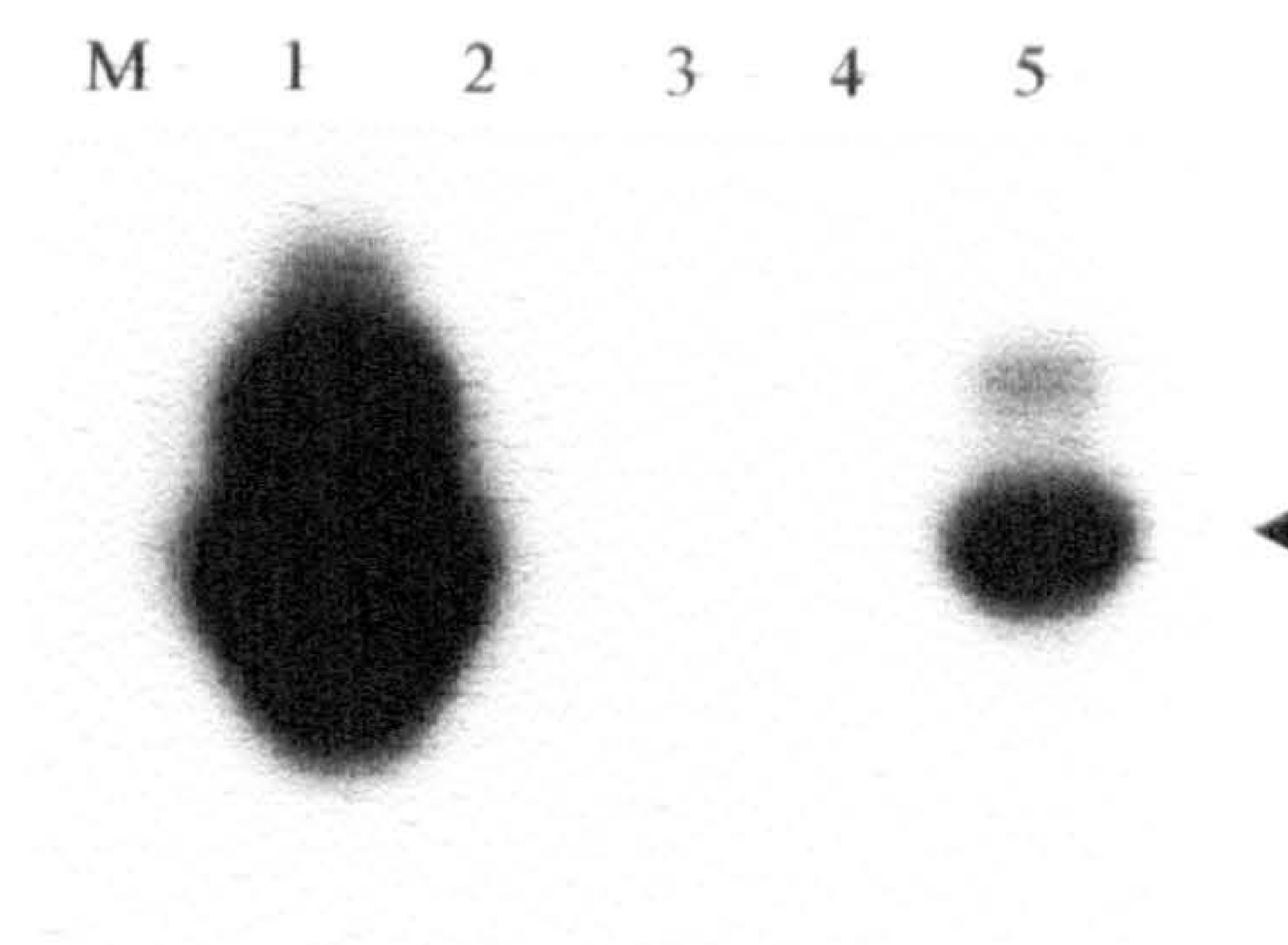
a.



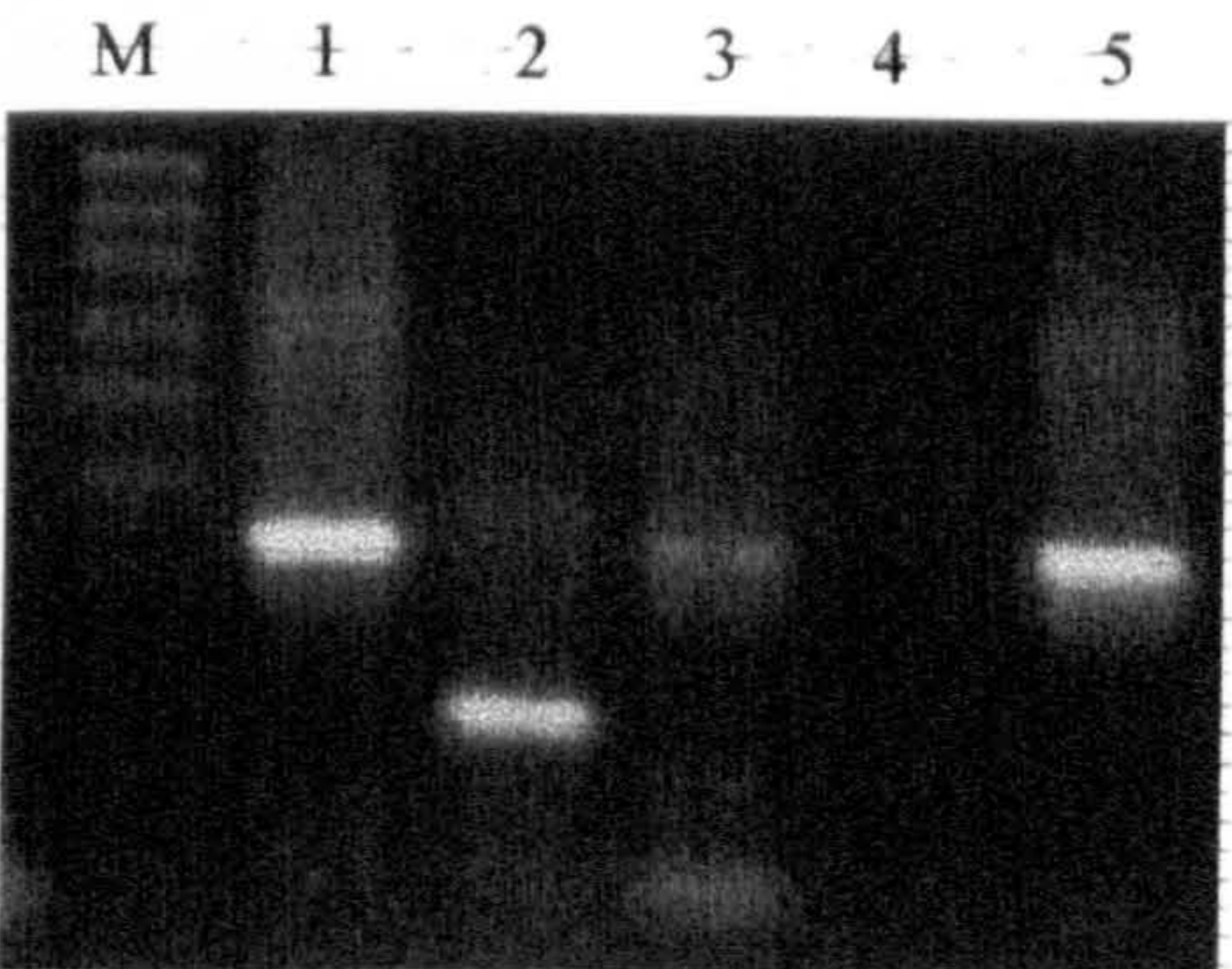
b.



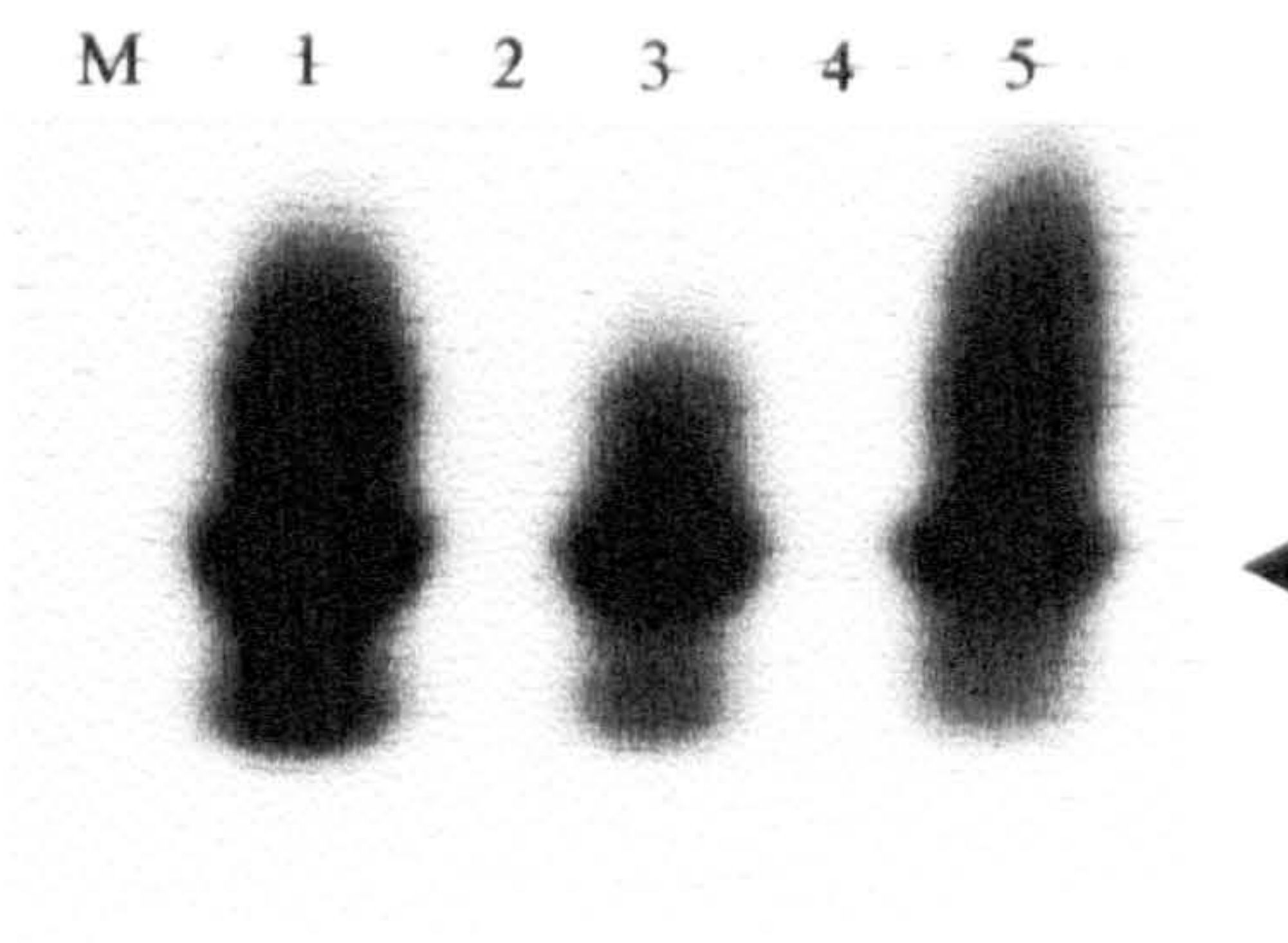
c.



d.



e.



f.

Legend for Figure 9.3.

(a) gel and (b.) Southern blot analysis of measles N-gene *rTth*-mediated RT-PCR products (section 4.2.8.) using PBMC RNA samples A to H (lanes A to H). Lane 9 contains products from a positive control reaction with 1 ng total RNA from measles-infected Vero cells and negative control reaction 10 contained water.

(c.) Gel and (d.) Southern blot analysis of measles N-gene *rTth*-mediated RT-PCR products. Lanes 1 and 5 contain products from positive control reactions with 10 and 1 ng, ~~receptively~~, of total RNA from measles virus-infected Vero cells. Lane 3 contains products from a reaction with 100 ng of total PBMC RNA from a measles vaccine recipient.

All RT-PCR products were probed with radiolabelled AB10 (section 4.2.9.).

(e.) Gel and (f.) Southern blot analysis of measles H-gene RT-nPCR products. RNA samples were the same as those described above (c. and d.). RT-PCR products were probed with radiolabelled H5 (section 4.2.9.).

Lanes M contain a 100 bp DNA size marker (Table 4.3.).

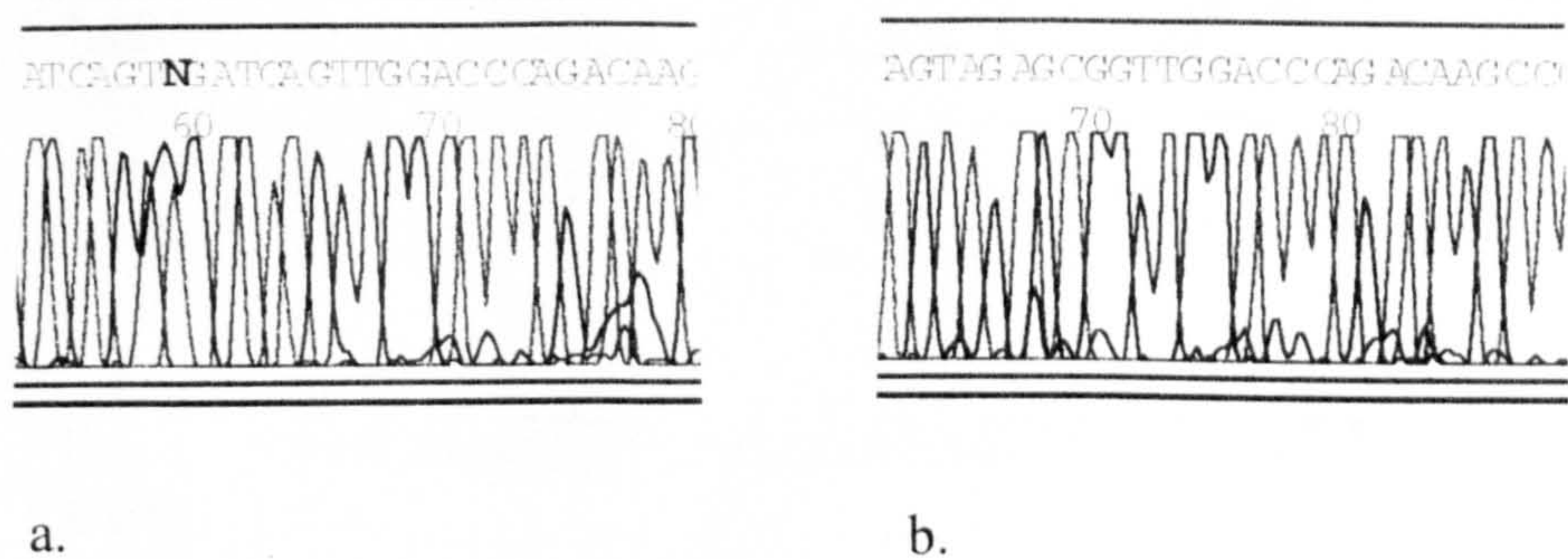
9.2.3. Sequence analysis of PCR products.

Wild type measles N-gene RT-PCR products derived from intestinal samples 3a and 11b, and PBMC sample A, were gel-purified (section 4.2.8.) and in order to generate appropriate amounts of PCR products for direct, automated sequencing, were subsequently reamplified using a semi-nested PCR with *Taq* DNA polymerase and primers MV3 and MV5 (section 4.2.5.). These re-amplified PCR products were gel-purified (section 4.2.11.) and sequenced directly using an automated DNA sequencer (4.2.15.). An example of an automated sequencing result is shown in Figure 9.4.

Measles H- and N-gene RT-PCR products from SSPE RNA, and measles H-gene RT-PCR products from the vaccine recipient PBMC RNA sample, were gel purified (section 4.2.11.) and used for direct, automated sequencing as described in section 4.2.15.

Sequencing of RT-PCR products from samples 3a, 11b, and A showed that they all possessed identical sequences to that of the laboratory Hu2-strain virus. Full sequences for measles H- and N-gene PCR products from SSPE RNA samples are given in Figures 9.5. and 9.6., respectively.

Figure 9.4: Sequence analysis of measles PCR products.



Sequence data generated from PCR products (section 4.2.15.) from reactions containing SSPE RNA sample 1 (a.) and a laboratory Hu2 strain contaminant in sample 3a (b.). Measles virus Hu2-strain can be identified by the substitution of A for G at position 1336 of the measles virus genome.

Figure 9.5: Measles N-gene sequences from SSPE RNA samples. Letters in superscript represent amino acid changes.

	⇒D	Reading Frame
Con	TGCCGAGGATGCAAGGCTTGTTTCAGAGATTGCAATGCATACT	
SSPE1	
SSPE2	
SSPE3C ^{D→D}
SSPE4A ^{L→I}
Con	ACTGAGGACAGGATCAGTAGAGCGGTTGGACCCAGACAAGCC	
SSPE1A ^{A→A}
SSPE2A ^{R→K}
SSPE3	
SSPE4	
Con	CAAGTGTCATTTCTACACGGTGATCAAAGTGAGAATGAGCTA	
C		
SSPE1A ^{E→E}
SSPE2	
SSPE3	
SSPE4A ^{E→E}
Con	CGAGATTGGGGGGCAAGGAAGATAGGAGGGTCAAACAGAGT	
C		
SSPE1G ^{L→W}T ^{R→M}
SSPE2	
SSPE3	...A ^{P→P}
SSPE4G ^{L→W}T ^{R→M}
Con	GAGGAGAAGCCAGGGAGAGCTACAGAGAAACCGGGCCCAGC	
SSPE1T ^{S→S}	
SSPE2A ^{A→D}
SSPE3T ^{P→S}
SSPE4T ^{S→S}	

Figure 9.6: Measles H-gene sequences from SSPE RNA samples. Letters in superscript represent amino acid changes.

	⇒Reading Frame
Con	ACGGGGAAGATTCTATCACAATTCCCTATCAGGGATCAGGGA
A	
SSPE1
SSPE2	...T ^{H→H}
SSPE3T ^{S→S}
SSPE4
Con	AGGTGTCAGCTTCCAGCTCGTCAAGCTAGGTGTCTGGAAATCC
SSPE1
SSPE2
SSPE3
SSPE4
Con	CCAACCGACATGCAATCCTGGGTCCCCTTATCAACGGATGATC
SSPE1
SSPE2
SSPE3
SSPE4
Con	CAGTGATAGACAGGCTTTACCTCTCATCTCACAGAGGTGTTAT
SSPE1A ^{V→V}
SSPE2
SSPE3
SSPE4
Con	CGCTGATAATCAAGCAAAATGGGCTGTCCCGACAACACGAAC
SSPE1A ^{R→R}
SSPE2
SSPE3
SSPE4	T ^{I→I}C ^{D→D}A ^{R→R}
Con	AGATGACAAGTTGCGAATGGAGACATGCTTCCAACAGGCGTG
SSPE1G ^{Q→Q}
SSPE2G ^{Q→Q}
SSPE3G ^{Q→Q}
SSPE4G ^{Q→Q}
Con	TAAGGGTAAAATCCAAGCACTCTGCGAGAA
SSPE1
SSPE2
SSPE3
SSPE4

9.4. DISCUSSION

Good quality total RNA was successfully isolated from PBMCs, SSPE material and intestinal resection tissue, as assessed by *rTth*-mediated RT-PCR for U1A RNA and agarose gel electrophoresis. Internal modified control transcripts were successfully amplified from intestinal RNA samples, implying that the measles RNA isolation procedure had not been destructive to measles RNA.

Due to low yields of PBMC RNA, the concentration of PBMC RNA could not be accurately determined by spectrophotometry and 5 µl of PBMC RNA was used for gel analysis and RT-PCR experiments.

In experiments with SSPE clinical samples, measles RNA was detected in all brain tissues tested confirming the ability of the methods described to detect measles RNA in persistently infected tissue. Indeed, as for NASBA (Chapter 6) measles RNA was detected in one tissue sample in the apparent absence of measles nucleoprotein (determined by immunohistochemistry), emphasising the increased sensitivity of RT-PCR over immunohistochemistry.

ed Measles virus RNA was not detected in PBMCs of patients with SSPE. This concords with the findings of Schneider-Schaulies *et al.* (1991). This result could have occurred because of either a). measles virus RNA being present below the sensitivity limits described, or b). persistent measles virus was not present in the peripheral blood of the SSPE patients.

In experiments to determine the presence of measles RNA in PBMCs of a vaccine recipient, measles H-gene was detected and sequence analysis confirmed the vaccine recipient PBMC RNA RT-PCR product to be from the AIK-C strain of measles virus (data not shown). The inability to detect measles virus N-gene RNA from a clinical positive control sample may, however, reflect specific problems associated with the extraction of measles virus N-gene RNA observed by others (D. Griffin, Johns Hopkins University, Baltimore, USA; personal communication).

Due to the small amount of RNA from the vaccine recipient PBMC, the results of these experiments could not be repeated. It remains possible that the vaccine recipient PBMC RNA sample was contaminated with measles H-gene PCR products, especially as contamination problems have been reported in the

laboratory in which this PBMC RNA was generated (H. Kawashima, Tokyo Medical School, Japan; personal communication).

In experiments to determine the presence of measles RNA in intestinal and PBMC samples from IBD and control patients, measles N-gene was detected in intestinal tissue samples 3a and 11b, and PBMC sample A.

Sequence analysis experiments showed that RT-PCR products arising from SSPE RNA had not arisen as a result of PCR contamination with the laboratory Hu2-strain of measles virus. Sequence analysis of the *rTh*-mediated RT-PCR products from samples 3a, 11b and A showed that they possessed identical sequences to laboratory strain (Hu2) virus, suggesting that they had arisen as a result of contamination of *rTh*-mediated RT-PCR reactions with laboratory Hu2-strain measles RNA. This was supported by the fact that duplicate reactions tested negative for measles N-gene RNA. Also, none of the IBD and control samples tested were positive for measles H-gene RNA. The presence of PCR contamination could be unequivocally demonstrated by the sequencing of measles N-gene PCR products and analysis for a guanine substitution of adenine at position 1336 - characteristic of the measles laboratory Hu2 strain sequence. Therefore, artifactual results arising from PCR contamination were not misinterpreted as resulting from measles virus-infected clinical samples.

In summary, other than as a contaminant, measles virus RNA was not detected in any sample from IBD or control patients in the experiments reported in this investigation. These results are discussed in detail in Chapter 11.

Chapter 10

Chapter 10: ANALYSIS OF CLINICAL SAMPLES FROM PATIENTS WITH AUTISTIC ENTEROPATHY FOR THE PRESENCE OF MEASLES, MUMPS AND RUBELLA RNA USING RT-PCR

10.1. INTRODUCTION

Immunohistochemistry experiments have suggested the presence of measles nucleoprotein in biopsy samples from patients with autistic enteropathy (Wakefield *et al.* 1998a). Twenty two affected children were used for this study. The mean age was 5 years 6 months and the mean time between MMR vaccination and onset of symptoms (behavioural or physical) was 3 weeks for 14 of the children. Symptoms of two children predated their MMR vaccination and information was not available for the remaining six children. PBMCs from 6 age-matched controls were also used in this study.

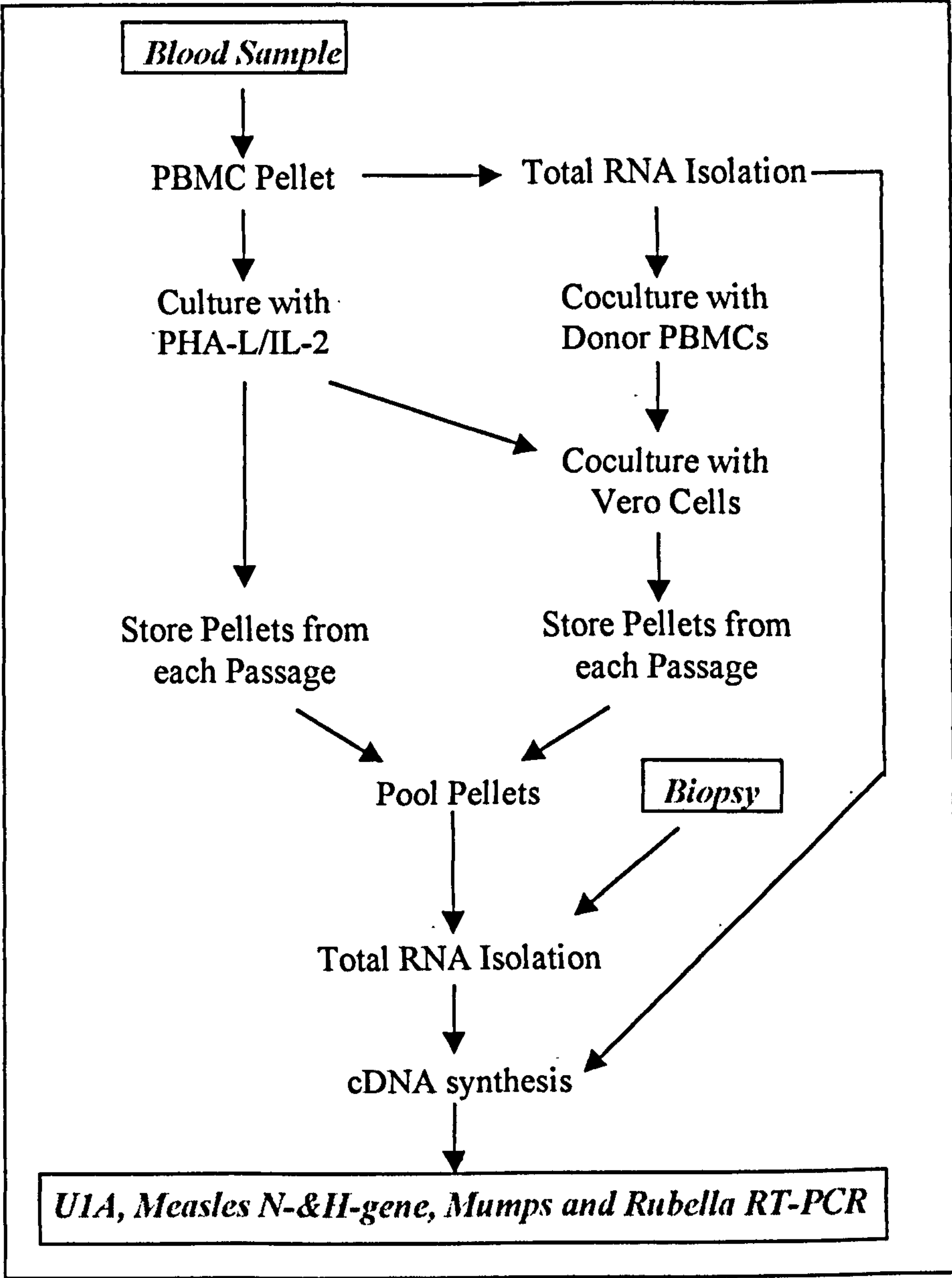
It was decided to test blood and biopsy samples (section 4.2.3.) from these patients for the presence of both measles N- and H-gene by RT-PCR. In addition, since MMR vaccination may have been a factor leading to the development of autistic enteropathy (Wakefield *et al.* 1998b), it was also decided to test biopsy and blood samples from these patients for the presence of mumps and rubella RNA. Verbal consent was obtained from all affected and control patients (and their parents) providing blood samples for this study. Ethics approval was obtained from the Royal Free Hospital Ethics Committee.

Because of the low amounts of total RNA which could be isolated from biopsy and PBMC samples, it was decided to generate a random pool of cDNAs from each RNA sample for subsequent PCR analysis of a number of templates.

Cocultivation experiments were performed in this study for the isolation and propagation of measles, mumps and rubella viruses. Cocultivation of PBMCs with Vero cells may permit viral replication and hence enhance the chance of detecting measles, mumps and rubella viruses (Forthal *et al.* 1992 and 1993). It was therefore decided to cocultivate PBMC samples with Vero cells and use total RNA isolated from cocultures for RT-PCR for measles, mumps and rubella RNA.

The full strategy of viral RNA detection is represented in Figure 10.1.

Figure 10.1: Strategy of measles, mumps and rubella RNA detection in clinical samples of patients with autistic enteropathy.



For measles RNA detection experiments, total RNA from SSPE autopsy material (described in the previous chapter) was used as a control to test the RT-nPCR methods ability to detect measles RNA in persistently infected tissue. Total RNA from measles-infected Vero cells (described in Chapter 5) was also used as a positive control.

For mumps and rubella detection experiments, positive control mumps RNA was obtained by isolation of total RNA from the supernatant of Vero cells infected with the VR 106 strain of mumps virus and positive control rubella RNA was obtained by isolation of total RNA from Vero cells infected with the M33 strain of rubella virus (Table 4.2.).

Aims. The aims of this study were to:

1. Cocultivate PBMCs from patients with autistic enteropathy with Vero cells in order to propagate any measles, mumps and rubella viruses present, thereby increasing the level of viral RNA.
2. Isolate total RNA from biopsies, PBMCs and Vero cell cocultures from patients with autistic enteropathy.
3. Analyse RNA samples for the presence of measles, mumps and rubella RNA using RT-PCR.

10.2. RESULTS.

10.2.1. Total RNA isolation and analysis of integrity.

Vero cell cocultures. PBMCs from 10 affected and one control patient were isolated from 10 ml blood and used for cocultivation with Vero cells (section 4.2.2.). Subsequently, cocultures were pooled, coded and total RNA isolated (section 4.2.3.).

To assess the quality of RNA, 5 µl was used for RT (with random hexamers and MMLV reverse transcriptase) followed by PCR with *Taq* DNA polymerase and primers U1A1 and U1A2 (section 4.2.6.). Reaction products were analysed by agarose gel electrophoresis and hybridisation with a radiolabelled probe (U1A3; section 4.2.9.). In addition, 5 µl of total RNA was analysed by agarose gel electrophoresis (section 4.2.8.).

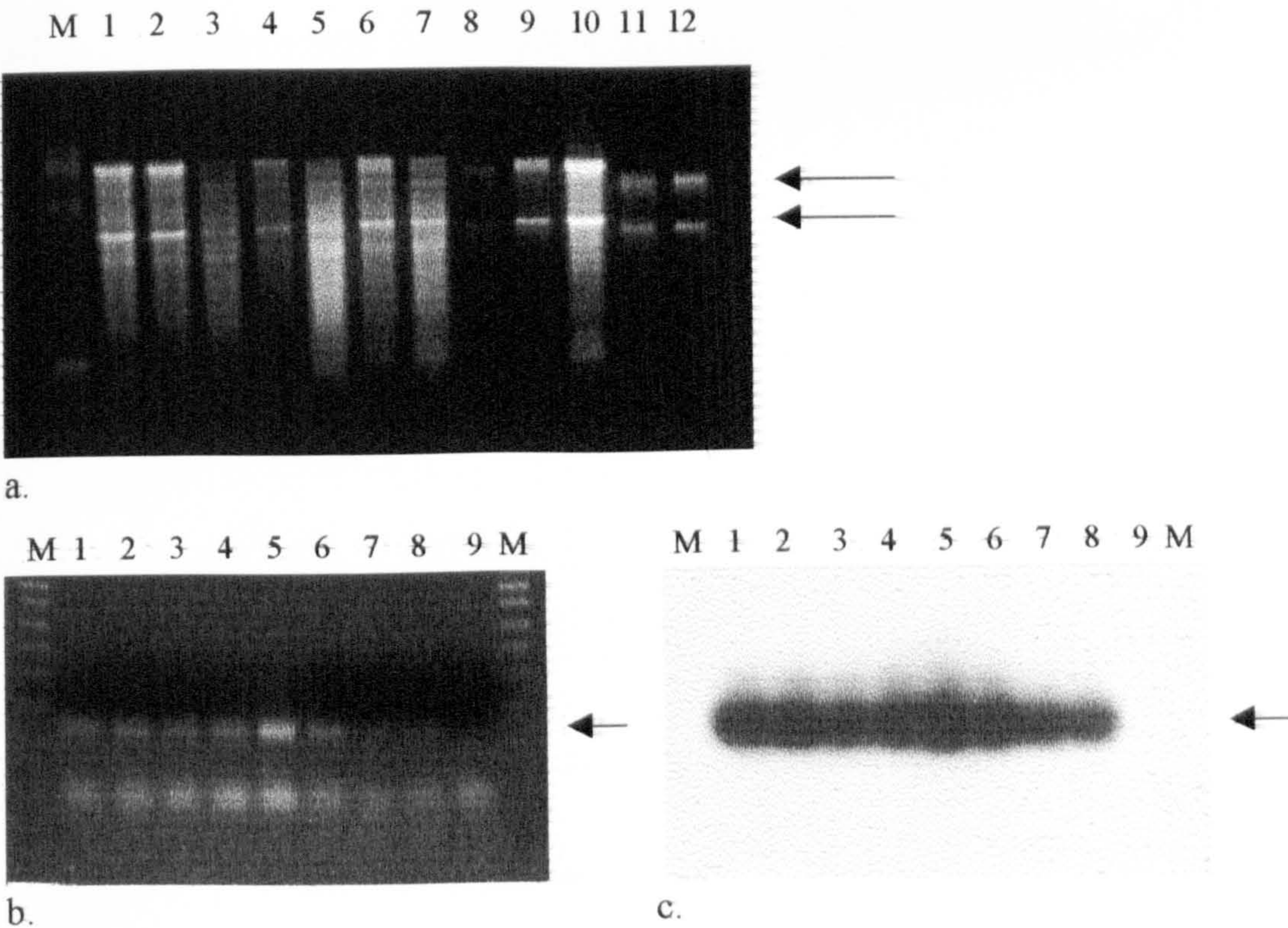
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Biopsy samples. Coded, fresh-frozen biopsy samples from 10 affected patients were used for total RNA isolation (section 4.2.3.). RNA integrity was assessed as described above.

Blood samples. PBMCs were prepared from 10 ml of blood from 12 affected and 6 controls patients (section 4.2.3.) and used for total RNA isolation (section 4.2.3.). RNA integrity was assessed as described above.

Following agarose gel electrophoresis, 28S and 18S RNA band were clearly visible for all biopsy, PBMC and Vero cell coculture RNA samples. In addition, all samples yielded positive results following RT-PCR for U1A RNA. _

Figure 10.2: Analysis of RNA quality



(a.) Representative agarose gel analysis (section 4.2.8.) of total RNA from Vero cell cocultures (lanes 1 to 12). Lane M contains an RNA size marker (0.24-9.5 nt; described in Table 4.3.).

(b.) Representative gel and (c.) Southern blot analysis of U1A PCR products, using *Taq* DNA polymerase (section 4.2.5.), from total RNA from biopsy samples (lanes 1 to 8). Lane 9 contains products from a negative control reaction with water. PCR products were probed with radiolabelled U1A3 (section 4.2.9.). The arrows point to a 225 bp U1A PCR product. Lane M contains a 100 bp DNA size marker (Table 4.3.).

10.2.2. Detection of measles, mumps and rubella RNA.

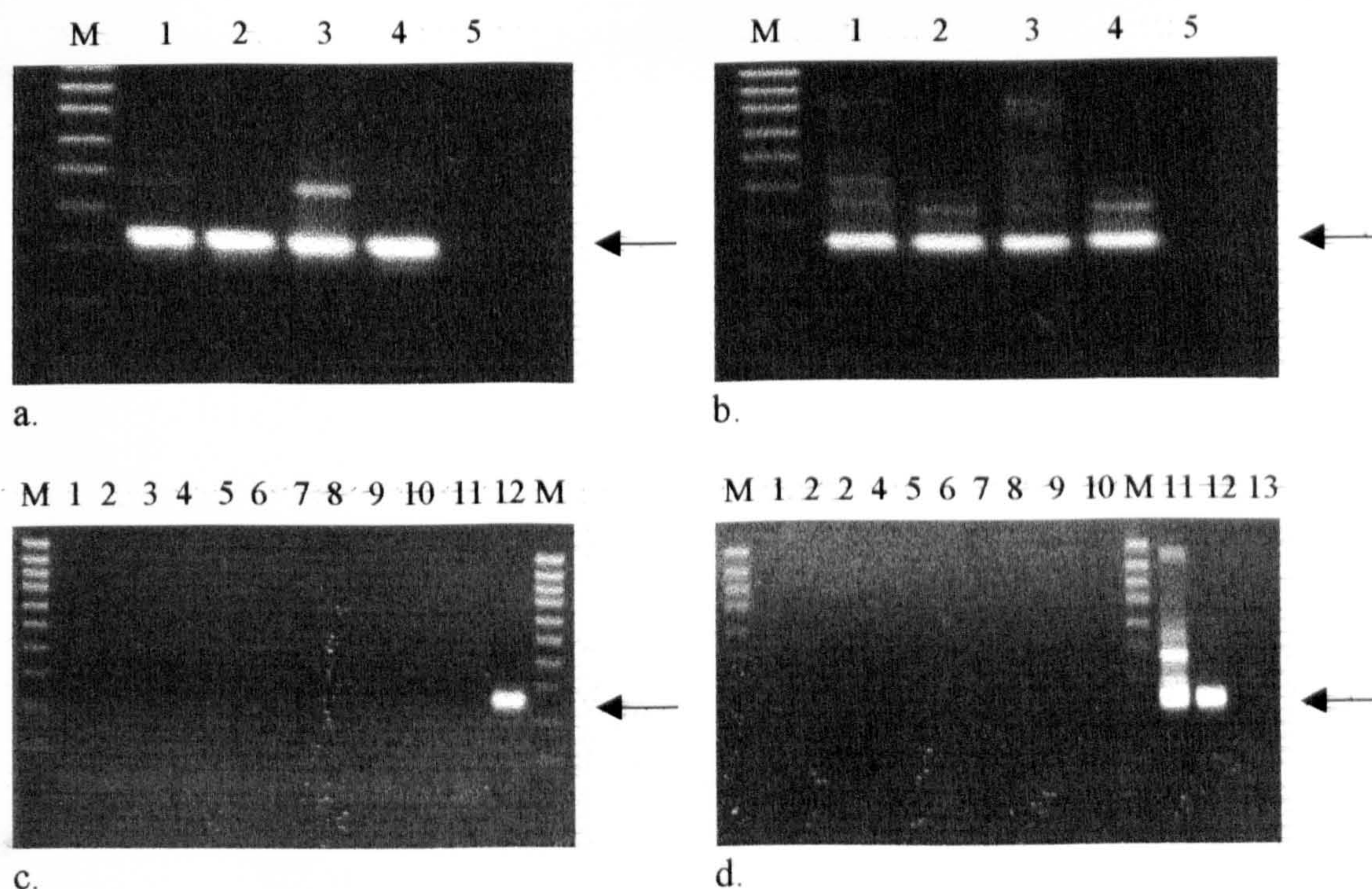
RT-PCR reactions. cDNA was generated from 5 µl of each total RNA sample using MMLV reverse transcriptase and random hexamers (section 4.2.3.) and 1 µl of cDNA used for PCR detection (with *Taq* DNA polymerase) of measles, mumps or rubella cDNA templates (section 4.2.5). Reaction details are as follows

1. Measles N-gene cDNA was detected in a nested reaction containing primer pairs MV1 and MV2 (first round), then MV3 and MV4 (second round). Reaction products were analysed by gel electrophoresis (section 4.2.8.).
2. Measles H-gene cDNA was detected in a nested reaction containing primer pairs HH3 and H6 (first round), then H4A and H7 (second round). Reaction products were analysed by gel electrophoresis (section 4.2.8.).
3. Mumps phosphoprotein gene cDNA was detected using primer pairs MUP1 and MUP2 based on a method described by Ni *et al.* (1997). Reaction products were analysed by gel electrophoresis (section 4.2.8.) and hybridisation with a radiolabelled probe (MUP3; section 4.2.9.).
4. Rubella E1 gene cDNA was detected in a nested reaction containing primer pairs R2 and R7 (first round), then R11 and R8C (second round), based on a method described by Bosma *et al.* (1995). Reaction products were analysed by gel electrophoresis (section 4.2.8.).

Analysis of cDNA samples for measles N- and H-gene sequences by nPCR (Figure 10.3.). Measles N-gene RNA was detected in the Vero coculture RNA derived from patients 2, 3, 16, and 21, and in the biopsy RNA from patient 7. Sequence analysis of PCR products from these samples showed that they contained identical sequences to the laboratory strain (Hu2) virus.

cDNA from 100 ng of total RNA from SSPE brain material gave positive PCR results for measles N- and H-gene analysis. Sequence analysis of these PCR products confirmed that they had not arisen as a result of PCR contamination.

Figure 10.3: Analysis of cDNA from autistic enteropathy and SSPE



(a.) Gel analysis of measles N-gene nPCR products (section 4.2.5.) using cDNA derived from 100 ng SSPE RNA (lanes 1 to 4). Negative control reaction 5 contained water. The arrow points to a 253 bp PCR product

(b.) Gel analysis of measles H-gene nPCR products using cDNA derived from 100 ng SSPE RNA (lanes 1 to 4). Lane 5 contains products from a negative control reaction with water. The arrow points to a 335 bp PCR product.

(c.) Representative gel analysis of measles N-gene nPCR products from cDNA derived from total PBMC RNA samples 1 to 10 (lanes 1 to 10). Lane 11 contains products from a negative control reaction with water and lane 12 contains products from positive control reaction with 1 ng total RNA from measles-infected Vero cells. The arrow points to a 253 bp PCR product.

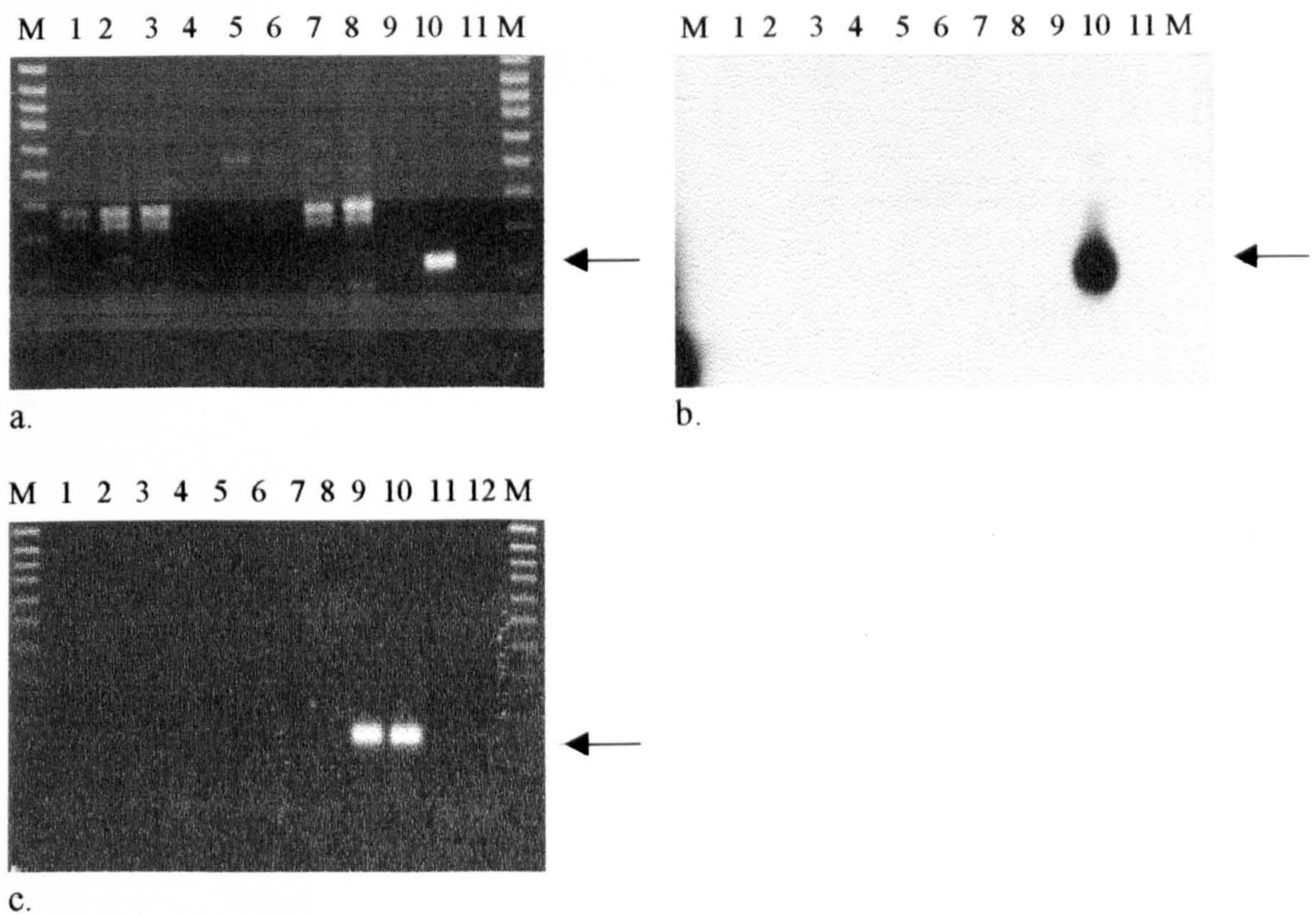
(d.) Representative gel analysis of measles H-gene nPCR products from cDNA derived from total PBMC RNA samples 1 to 10 (lanes 1 to 10). Lanes 11 and 12 contains products from positive control reactions with 10 ng and 1 ng of total RNA from measles-infected Vero cells, respectively. Lane 13 contains products from a negative control reaction with water.

Lanes M contain a 100 bp DNA size marker (Table 4.3.).

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PCR for mumps and rubella cDNA (Figure 10.4.). In all experiments, positive control mumps and rubella cDNA samples yielded the expected PCR products. ~~None~~¹ samples analysed gave positive results for either mumps or rubella cDNA. A full summary of measles, mumps and rubella RT-PCR results and the samples used is given in Table 10.1.

Figure 10.4: Analysis of cDNA from autistic enteropathy clinical samples for mumps and rubella RNA using PCR.



(a.) Representative gel and (b.) Southern blot analysis of mumps PCR products (section 4.2.5.) from cDNA derived from Vero cell cocultures (lanes 1 to 10). Positive control reaction 10 contained cDNA derived from 1 ng of total RNA from mumps-infected cell cultures. Lane 11 contains products from a negative control reaction with water. PCR products were probed with radiolabelled MUP3 (section 4.2.9.). The arrows point to a 223 bp PCR product.

(c.) Representative gel analysis of rubella nPCR products from cDNA derived from Vero cell cocultures (lanes 1 to 8). Lanes 9 and 10 contains products from positive control reactions with cDNA derived from 1 ng total RNA from rubella-infected cell cultures. Lanes 11 and 12 contains products from negative control reactions with water.

The arrow points to a 142 bp PCR product. Lanes M contain a 100 bp DNA size marker (Table 4.3.).

Table 10.1: U1A, measles, mumps and rubella RT-PCR results using biopsies, PBMCs and Vero cell cocultures. Bx = biopsy. C = contaminant.

	Sample	U1A	MV-N	MV-H	Rubella	Mumps
Affected patients						
1	Bx	✓	✗	✗	✗	✗
2	Bx	✓	✗	✗	✗	✗
	Cultures	✓	✓(C)	✗	✗	✗
3	Bx	✓	✗	✗	✗	✗
	PBMCs	✓	✗	✗	✗	✗
	Cultures	✓	✓(C)	✗	✗	✗
4	Bx	✓	✗	✗	✗	✗
	PBMCs	✓	✗	✗	✗	✗
	Cultures	✓	✗	✗	✗	✗
5	Bx	✓	✗	✗	✗	✗
6	Cultures	✓	✗	✗	✗	✗
7	Bx	✓	✓(C)	✗	✗	✗
	PBMCs	✓	✗	✗	✗	✗
8	Cultures	✓	✗	✗	✗	✗
9	Bx	✓	✗	✗	✗	✗
10	Cultures	✓	✗	✗	✗	✗
11	Bx	✓	✗	✗	✗	✗
	PBMCs	✓	✗	✗	✗	✗
12	Cultures	✓	✗	✗	✗	✗
13	Bx	✓	✗	✗	✗	✗
	PBMCs	✓	✗	✗	✗	✗
	Cultures	✓	✗	✗	✗	✗
14	PBMCs	✓	✗	✗	✗	✗
15	Bx	✓	✗	✗	✗	✗
16	PBMCs	✓	✗	✗	✗	✗
	Cultures	✓	✓(C)	✗	✗	✗
17	PBMCs	✓	✗	✗	✗	✗
18	PBMCs	✓	✗	✗	✗	✗
19	PBMCs	✓	✗	✗	✗	✗
20	PBMCs	✓	✗	✗	✗	✗
21	Cultures	✓	✓(C)	✗	✗	✗
22	PBMCs	✓	✗	✗	✗	✗
Control patients						
23	PBMCs	✓	✗	✗	✗	✗
24	PBMCs	✓	✗	✗	✗	✗
25	PBMCs	✓	✗	✗	✗	✗
26	PBMCs	✓	✗	✗	✗	✗
	Cultures	✓	✗	✗	✗	✗
27	PBMCs	✓	✗	✗	✗	✗
28	PBMCs	✓	✗	✗	✗	✗

10.3. DISCUSSION

Good quality RNA was isolated from biopsies, PBMCs and Vero cell cocultures as determined by agarose gel electrophoresis and RT-PCR for U1A RNA.

SSPE RNA samples yielded positive results in both measles N- and H-gene RT-nPCR experiments. Sequence analysis of PCR products confirmed that they had not arisen as a result of PCR contamination. Positive control mumps and rubella RNA samples yielded positive results for mumps RT-PCR and rubella RT-nPCR experiments, respectively. These results indicated that the methods employed were capable of detecting measles, mumps and rubella RNA within total RNA samples.

In experiments to determine the presence of measles, mumps or rubella RNA in clinical samples, four Vero cell coculture samples and one biopsy sample tested positive for measles N-gene RNA. However, sequence analysis of these PCR products showed that they were identical to that of the laboratory Hu2-strain of measles virus. These results almost certainly arose from PCR contamination. No measles H-gene RNA, mumps RNA or rubella RNA was detected in any of the RNA sample from biopsies, blood or Vero cell cocultures.

It is possible that persistent measles, mumps or rubella virus may be defective and this may prevent their efficient isolation and propagation. This is the case with SSPE (Carter and ter Meulen, 1990). Reasons for the lack of detectable measles, mumps and rubella RNA in these samples and the implications of these results are discussed in detail in Chapter 11.

Chapter 11

Chapter 11: GENERAL DISCUSSION

11.1. MEASLES VIRUS RNA AND IBD

The negative measles NASBA and RT-PCR results described in Chapters 6 and 9, respectively, support the results of previously published work from our group and others (Smith, 1994; Lewey *et al.* 1995; Iizuka *et al.* 1995; Fan *et al.* 1996; Haga *et al.* 1996). Although there may have been problems with these studies in terms of detection assay sensitivity, the reproducible lack of detectable measles RNA in intestinal tissue is striking nonetheless.

Given the two sets of results in Chapters 6 and 9, there are three clear possibilities as to why measles RNA was not detected in IBD clinical samples: measles virus is not present; measles antigens are present in the absence of measles RNA; measles RNA is present but not detectable using the methods described in this or other studies.

11.1.1. Measles virus is not present

Such an explanation would be in keeping with the results described here and also with previously published measles RT-PCR results for IBD samples (Smith, 1994; Lewey *et al.* 1995; Iizuka *et al.* 1995; Fan *et al.* 1996; Haga *et al.* 1996).

However, it is possible that early exposure to measles virus may lead to the later development of IBD without any need for viral persistence, therefore the positive epidemiological association is not dependent upon the presence of measles virus in IBD clinical samples. At least three mechanisms could account for this possibility: molecular mimicry leading to autoimmunity; immunosuppression leading to persistence of other infectious agents; and immune dysfunction.

Autoimmunity. As discussed in Chapter 3, exposure to measles virus leads to lifelong immunity. Antibody cross-reactivity between measles antigens and self antigens may lead to the propagation of an autoimmune reaction. Self-antigens may be expressed later in life in response to a genetic or environmental trigger which are recognised by memory B-cells specific for measles antigens. If, for

example, these antigens are expressed on intestinal submucosal endothelial cells, an autoimmune reaction may occur leading to the generation of granulomatous vasculitis and chronic inflammation. Therefore it may be interesting to test the cross-reactivity of auto-antibodies detected in IBD patients with measles virus antigens.

An autoimmune reaction against myelin basic protein, following measles virus infection has been described previously in Lewis rats (Liebert *et al.* 1988) and this reaction may be involved with the pathogenesis of diseases associated with persistent measles virus infection such as SSPE. Host cell myelin basic protein is believed to be involved with measles virus replication and is a component of viral particles which are recognised by the host immune system (Wirguin *et al.* 1993).

Interestingly, T cells reactive to, and B cells producing antibodies against (Link *et al.* 1992; Jingwu *et al.* 1991), both myelin basic protein and measles virus have been demonstrated in the cerebrospinal fluid of patients with multiple sclerosis. These observations raise the possibility that a measles virus infection of the CNS is cleared by the host immune system but later triggers an autoimmune reaction, leading to chronic inflammation. This would account for the lack of measles RNA in multiple sclerosis tissue as determined by RT-PCR (Godec *et al.* 1992).

Early exposure to measles virus may be important in the generation of auto-antibodies and this could account for epidemiological associations between early measles virus exposure and IBD without a requirement for the presence of measles virus at the time of disease development.

Immunosuppression. As discussed in Chapter 3, a period of immune suppression follows both acute measles virus infection and measles vaccination. During this transient period of immunodeficiency, infectious agents in the gut lumen may be able to establish a persistent infection in intestinal submucosal endothelial cells without clearance by the host immune system. Such persistent agents may then trigger an abnormal immune response later in life, leading to the development of granulomatous vasculitis and chronic intestinal inflammation.

Immune dysfunction. Measles may be capable of disrupting the homeostasis of the gut immune system through the decreased production of IL-12 by follicular dendritic cells (Fugier-Vivier *et al.* 1998). IL-12 is known to induce the differentiation of Th0 CD4+ T cells to Th1 CD4+ T cells (Heufler *et al.* 1996) and therefore, disruption of IL-12 production may lead to chronic immune dysregulation which manifests itself as IBD many years after the original virus infection has been cleared.

In susceptible individuals, early measles virus infection may lead to a chronic Th1/Th2 cytokine imbalance which may not become apparent until later in life when exposure to a particular environmental agent(s) leads to an abnormal immune response and the development of chronic inflammation. Such a mechanism would be in keeping with the epidemiological association between early exposure to measles virus and the development of IBD.

Although these three explanations may account for the epidemiological association between early exposure to measles virus and IBD, significant discrepancies are introduced between the negative RT-PCR results of this and other studies, and the results of previous studies concerning the detection of paramyxovirus-like particles (Wakefield *et al.* 1993; Knibbs *et al.* 1993), measles antigens (Wakefield *et al.* 1993; Lewin *et al.* 1995; Miyamoto *et al.* 1995; Ekbom *et al.* 1996; Daszak *et al.* 1997; Wakefield *et al.* 1997) and measles RNA (Wakefield *et al.* 1993) in IBD tissue. Such explanations would cast doubt on the validity of the results from previous studies:

Electron microscopy. Two independent reports have been published concerning the detection of paramyxovirus-like particles in areas of granulomatous inflammation in Crohn's disease tissue using electron microscopy (Knibbs *et al.* 1993; Wakefield *et al.* 1993). Both reports described the presence of intranuclear and intracytoplasmic inclusion bodies containing arrays of tubular structures morphologically consistent with paramyxovirus nucleocapsids. Such a description may apply to a range of viruses other than measles and it is possible that intracellular macromolecules may also form similar structures. The non-specificity of this technique prevents any definitive conclusion as to the presence

or absence of measles virus particles in IBD tissue, although these results justified further immunological studies.

Measles nucleoprotein monoclonal antibody studies. A study at the Royal Free Hospital reported positive immunostaining for measles nucleoprotein in tissue samples from 13 of 15 patients with Crohn's disease but not in either two cases of intestinal tuberculosis, a granulomatous negative control (Wakefield *et al.* 1993). The antibody used for this study was a commercially available monoclonal antibody directed against measles nucleoprotein (MAS 182r). Even though this antibody was shown to produce positive staining in positive control SSPE tissue sections, it is possible that it cross-reacted with cellular components within intestinal tissue leading to false-positive results.

A recent study by Iizuka and Masamune (1997) has provided evidence supporting the possibility that MAS 182r cross-reacts with a cellular protein of human origin. Iizuka and Masamune replicated the immunohistochemistry results of Wakefield *et al.* (1993) using MAS 182r to show positive staining in Crohn's disease tissue. In order to confirm the specificity of antibody binding, cDNA from Crohn's disease tissue was used to construct a phage λ gt11-expression library. This library thus represented all the proteins encoded for by all the RNA molecules present in the Crohn's disease tissue.

The expression library was screened using MAS 182r and one positive clone identified. The DNA from this positive clone was sequenced and compared with known sequences in databases. This revealed that the DNA sequence was 99% homologous with the 3' terminus of a human gene of unknown function (accession number AA449055) and did not share any sequence homology with the measles nucleoprotein gene.

A second monoclonal antibody (4F12) directed against the protein encoded by this human gene sequence was produced and used to stain Crohn's disease tissue, revealing a similar staining pattern to that of MAS 182r, providing further evidence that MAS 182r cross-reacts with a protein of human origin. Therefore it would appear that MAS 182r recognises epitopes present on both measles nucleoprotein and the human protein which are structurally related but have no amino acid sequence similarity.

This finding brings into doubt the validity of the original MAS 182r staining for measles nucleoprotein in Crohn's disease tissue. The fact that this antibody apparently recognises a protein specific for Crohn's disease granulomas is interesting in itself as this protein may be involved with the pathogenesis of Crohn's disease.

The discovery of cross-reactivity between measles nucleoprotein and a human protein raises the possibility that "molecular mimicry" may occur, leading to the development of IBD later in life. Early exposure to measles virus leads to a polyclonal antibody response to measles virus, clearance of the virus and lifelong immunity. Within this polyclonal antibody population may be antibodies which cross-react with the human protein recognised by MAS 182r. Later in life, these antibodies may recognise the human protein expressed in the gut, leading to an autoimmune response and generating chronic inflammation. This possibility cannot be investigated until the human protein has been fully characterized.

Measles nucleoprotein polyclonal antibody studies. Following the monoclonal antibody study, a subsequent project used double staining to characterize the immune response directed against cells staining positive for measles nucleoprotein in Crohn's disease tissue using a polyclonal antibody (Wakefield *et al.* 1997). Recombinant measles nucleoprotein was expressed in an *E.coli* vector and the bacterial cell lysate used to raise a measles nucleoprotein polyclonal antibody response in rabbits. The unpurified sera thus produced was used directly on positive control SSPE tissue, measles virus infected appendix tissue, and Crohn's disease tissue sections. Again, positive staining was observed in positive control tissue and also in areas of granulomatous inflammation in Crohn's disease tissue.

The antibody used in this study was not affinity purified and it is therefore possible that antibodies directed against a range of antigens other than measles were present in the polyclonal sera. As recombinant *E.coli* lysates were used as a source of measles nucleoprotein, the polyclonal sera probably contained antibodies directed against *E.coli* antigens which are likely to be present in gut tissue, particularly in areas of active intestinal inflammation, as reviewed by

Burke (1997).

The measles polyclonal sera^u was found to give positive staining in *E.coli*-infected tissue and in cytopins of cultured *E.coli*, demonstrating the presence of antibodies directed against *E.coli* within this sera^u. However, staining measles antigen-positive Crohn's disease tissue, using an antibody specific for *E.coli* generated different patterns of signal, indicating that specific measles antibody-antigen interactions were present in tissue sections analysed (unpublished data).

Purification of recombinant measles nucleoprotein prior to immunisation (for instance, by immunoprecipitation) and affinity purification of this polyclonal antibody sera^u, may have reduced any problems regarding antibody specificity.

In addition, the polyclonal antibody sera^u directed against measles nucleoprotein may contain antibodies which cross-react with the human protein recognised by MAS 182r, as discussed above.

Using this same polyclonal antibody sera^u, Lui *et al.* (1995) described non-specific staining in all IBD and control tissues analysed. Although different methodologies may account for these strikingly different results, the presence of antibodies directed against non-measles antigens may be a significant factor in the result reported by Wakefield *et al.* (1997).

Measles matrix protein study. In support of the findings of Wakefield *et al.* (1993), Miyamoto *et al.* (1995) described the identification of a monoclonal antibody (mAb 86) directed against the measles matrix protein which generated positive immunofluorescence signals in a significantly greater number of Crohn's disease tissue than ulcerative colitis or control tissues. mAb 86 was shown to react with a protein within wild-type measles-infected Vero cells of approximately the same size as the measles matrix protein by Western blot analysis. This antibody did not give any staining with uninfected cells, rinderpest-infected cells or canine distemper virus-infected cells, indicating that it does not cross-react with other morbilliviruses.

The same antibody did not react with any antigens within HEL cells infected with an SSPE-strain of measles virus. As suggested by Miyamoto *et al.*, mAb 86 may have recognised wild-type measles matrix protein but not SSPE-strain of measles virus due to mutations accumulated during viral persistence affecting

the antigenicity of measles SSPE-strain matrix protein as described by Hirano *et al.* (1992).

The antigen with which mAb 86 reacted in measles-infected Vero cells could have been isolated (for instance, by immunoprecipitation) and, if possible, sequenced to confirm the specificity of mAb 86.

In the same study, a monoclonal antibody directed against measles nucleoprotein failed to generate any positive staining in Crohn's disease tissue.

Immunogold electron microscopy. Three studies reported positive staining in Crohn's disease tissue using immunogold electron microscopy (Lewin *et al.* 1995; Ekbom *et al.* 1996; Daszak *et al.* 1997). All these studies employed the same measles nucleoprotein polyclonal antibody used by Wakefield *et al.* (1997) and therefore the same problems concerning antibody specificity may have arisen, as discussed above. Positive signal was detected in the foci of a granuloma in one case of intestinal tuberculosis analysed, raising questions as to the specificity of the measles nucleoprotein polyclonal antibody sera^{um}. Alternatively, this tissue may have been persistently coinfectd with measles virus and tuberculosis.

Epidemiology. Several studies have provided epidemiological evidence for the involvement of measles virus in the aetiology of IBD, particularly Crohn's disease (Ekbom *et al.* 1994; Thompson *et al.* 1995a; Ekbom *et al.* 1996). Although controversial, the results suggest of these studies that early exposure to measles virus is a significant risk factor for the development of IBD. As discussed above, early exposure to measles virus and molecular mimicry, without virus persistence, may be a risk factor for the development of IBD later in life, accounting for the observed epidemiological associations.

11.1.2. Measles antigens are present in the absence of measles RNA

It may be possible that measles antigens are present in IBD tissue in the absence of measles RNA. Such a phenomenon would be in keeping with the positive immunohistochemical results and the negative RT-PCR results. Three explanations may account for the presence of measles antigen but not measles

RNA in IBD tissue: retention of viral antigen by cells exposed to measles virus; degradation of viral RNA during chronic inflammation; and the interesting possibility that latent measles DNA may be present.

Retention of viral antigens. As discussed later, even though the underlying mechanisms of antigen retention are not fully understood, antigens can be retained by cells for prolonged periods (Tew and Mandel, 1979). The life-span of a human endothelial cell is several years (Vanhoutte *et al.* 1995), therefore these cells may retain measles antigens for long periods of time following *in utero* or *post natal* measles virus infection. Several years later, an environmental or genetic trigger may give rise to MHC class I-restricted expression of these viral antigens (Dhib-Jalbut and Cowan, 1993), leading to monocyte adhesion and the development of granulomatous vasculitis. Therefore, upon analysis of IBD tissue, measles antigens may be detected in the absence of measles RNA.

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this explanation would be in keeping with the epidemiological association between early measles virus infection and the development of IBD.

Degradation of viral RNA. Early infection of intestinal submucosal endothelial cells with measles virus may lead to persistence of whole virus and trigger the development of granulomatous vasculitis later in life. The intestinal ischaemia, inflammation and infarction resulting from granulomatous vasculitis may lead to the degradation of labile RNA molecules but not more stable protein species. Therefore, by the time of intestinal tissue resection and analysis, only stable measles antigens may be present and detectable, whereas measles RNA molecules may have been degraded beyond the point where they are detectable by RT-PCR during chronic inflammatory processes.

On the other hand, measles RNA is readily detectable in SSPE tissue where chronic inflammation is responsible for much of the disease pathology (Poser, 1990), although the extent of inflammation is markedly different in IBD. Moreover, in this study measles RNA was detectable in SSPE autopsy material where the time between patient death and freezing of tissue was up to 24 hours, and during this time tissue autolysis may have been expected to degrade some of the RNA present. However, good quality RNA was isolated on the basis of gel

electrophoresis and housekeeping U1A RNA amplification. Moreover, measles RNA forms stable associations with measles nucleocapsids, rendering measles RNA highly resistant to nuclease digestion (Moyer *et al.* 1990).

In highly metabolic tissues, such as gut, more degradative processes may occur during chronic inflammation and following tissue resection, and this may lead to the degradation of small amounts of any measles RNA present.

Measles DNA. A recent publication by Klenerman *et al.* (1997) has identified lymphocytic choriomeningitis virus (LCMV) DNA in tissue from infected mice. This finding is highly significant with regard to measles virus since LCMV is a non-retroviral RNA virus. The authors decided to examine lymphoid tissue from infected mice for the presence of LCMV DNA since there was evidence of LCMV antigens in the absence of LCMV RNA following low-dose exposure to the virus. Moreover, infectious LCMV could not be isolated from tissue positive for LCMV antigens. The detection of LCMV DNA and antigens in the absence of LCMV RNA may be important in the context of the findings of measles nucleoprotein in Crohn's disease tissue in the apparent absence of measles RNA described in this study.

The reverse transcriptase activity required to synthesise LCMV DNA in infected cells may have arisen from three sources: co-infection with a retrovirus may have provided the necessary reverse transcriptase activity; endogenous reverse transcriptase activity may have arisen from retroviral DNA sequences present in the host genome; endogenous host enzymes capable of reverse transcriptase activity may have been responsible for the presence of LCMV DNA.

The authors of this study suggested that latent LCMV DNA may play an important role in long term immunity to LCMV. Latent LCMV DNA may function as a natural DNA vaccine - periodically transcribed and translated to generate antigens which boost the immune response to LCMV. In this manner, persistent LCMV DNA may be present in association with only transient levels of LCMV RNA. In general, protein has a longer half life than RNA *in vivo*, therefore LCMV antigens may be detected in the absence of LCMV RNA.

In 1975, a related study by Zhdanov (1975) reported the detection of integrated measles DNA in chronically-infected cell cultures, raising the possibility that

measles DNA may be a feature of measles virus persistence. However there are no published reports confirming these results using more sensitive molecular biology techniques now available. Moreover, there is no published data concerning measles DNA in persistent measles virus infections such as SSPE, although this lack of published data may indicate that measles DNA has not been sought in persistently infected tissue or cell cultures.

Interestingly, reverse transcriptase activity has been identified in cultures of chick embryo fibroblasts associated with an endogenous avian retrovirus (Weissmahr *et al.* 1997). These cells are used to produce the live, attenuated, measles vaccine and such reverse transcriptase activity has also been detected in MMR preparations themselves (Boni *et al.* 1996). These findings raise the possibility that prior to, or following vaccination, regions of measles RNA may be reverse transcribed to measles DNA and become integrated within the host genome, or persist in the cell cytoplasm. Periodic transcription and translation of these measles DNA sequences may then lead to expression of measles antigen, stimulating the production of measles antibodies and leading to prolonged immunity to measles virus. In this manner, measles antigen may be more readily detectable than transiently expressed measles RNA. Studies underway in several laboratories may determine whether latent measles DNA is produced in cells following measles vaccination.

If this phenomenon does occur, then it is possible that measles antigen expressed on host cells may be a target for the immune response, leading to chronic intestinal inflammation. The periodic expression of measles antigen would then account for the periods of remission and relapse observed in IBD. Transiently expressed measles RNA may be rapidly degraded and therefore measles RNA may not be present on examination of tissue samples in accordance with the results of this project.

The studies described in Chapters 6 and 9 used total RNA from IBD clinical samples to test for the presence of measles RNA and therefore the presence of measles DNA was not determined. RNA test samples were not purified by DNase digestion, therefore it is likely that these RNA samples were contaminated with cellular DNA. However, the small amount of cellular DNA contamination may have been insufficient to enable the detection of any measles

DNA present in the samples analysed.

11.1.3. Measles virus RNA is present but not detectable

Such an explanation would fit well with the positive immunohistochemical data and the epidemiological associations between measles virus and IBD. In previous studies to determine the presence of measles RNA in IBD tissue (Smith, 1994; Lewey *et al.* 1995; Iizuka *et al.* 1995; Fan *et al.* 1996; Haga *et al.* 1996), accurate quantitation of the detection assay employed was not performed. Since hybrid capture has been shown to improve the sensitivity of measles RNA detection 100-fold, it is likely that the sensitivity limits of the measles RNA detection methods described in previous studies was in the region of 10^6 measles RNA molecules. Therefore, the methods used in these studies may not have been capable of detecting low copy-number measles RNA. The detection sensitivity of the method employed in Chapter 9 was quantified by adding known numbers of a measles RNA transcript to intestinal tissue or PBMC homogenates prior to RNA extraction, hybrid capture and RT-PCR for measles RNA.

The efficiency of this method, and that of the NASBA method used in Chapter 5, was further confirmed by performing RNA amplification on a variety of measles RNA templates: total RNA from Hu2 strain measles virus-infected Vero cells; total RNA from a Schwarz strain measles vaccine sample; synthetic wild-type measles RNA transcripts; and total RNA from autopsy material of four cases of SSPE. Amplification of measles RNA from SSPE autopsy material demonstrated the ability of the methods used in Chapters 6 and 9 to detect a persistent measles virus infection. Moreover, the detection of measles RNA by RT-PCR and NASBA in one case with negative immunohistochemical staining demonstrated the advantage of these methods over immunohistochemistry for the detection of persistent measles RNA. The detection of measles virus RNA but not antigens in SSPE tissue has been demonstrated previously by *in situ* hybridisation (Allen *et al.* 1996). It is therefore unlikely that a persistent measles virus infection is detectable by immunohistochemistry but not by more sensitive nucleic acid amplification techniques.

Detection sensitivity. As a positive control, RT-PCR and NASBA were used to amplify a housekeeping RNA species - comprising part of the spliceosome subunit, U1A - present in all eukaryotic cells. The detection of U1A RNA gives an indication of the integrity of RNA but does not necessarily mean that any measles RNA present should be detectable, since only a very small proportion of cells may be infected with measles virus.

It is possible that measles RNA was present below the sensitivity limits of the measles RNA detection assays used in this study (as few as 10^4 measles N-gene RNA molecules). Measles virus may persist in only a very small number of intestinal microvascular endothelial cells, leading to focal granulomatous vasculitis and widespread chronic inflammation later in life. Immunogold electron microscopy data suggests a very low copy-number persistent measles virus infection in the foci of granulomatous inflammation in Crohn's disease tissue, in contrast to the relatively high copy-number infection observed in SSPE tissue (Lewin *et al.* 1995; Daszak *et al.* 1997). Such a focal, low-copy number infection may be difficult to detect even using highly sensitive molecular amplification methodologies.

However, as described in Chapter 8, 10^4 copies of a measles RNA transcript could be detected in tissue homogenates using hybrid capture followed by RT-PCR. In 1984, Lund *et al.* published the results of a study showing that up to 13,000 measles nucleocapsids are present per measles virion. If each nucleocapsid contains a measles RNA genome, then approximately 10^3 measles RNA molecules are present per measles virion and so, in theory, the presence of very few functional virus particles (possibly as little as 10) can be detected by this method. Because of this high level of sensitivity, it would seem unlikely that every IBD sample analysed contained measles RNA at a level below that of the sensitivity of the detection method described. On the other hand, measles virus may be present in Crohn's disease tissue as low copy-number defective viral particles rather than functional replicative virus and may therefore be difficult to detect.

Measles virus RNA isolation. The efficiency of measles RNA isolation may be below that of other RNA species, leading to an under-representation of measles

RNA in a total RNA sample. During total RNA isolation high concentrations of guanadinium thiocyanate salts are used for lysis, denaturation, and dissociation nucleoprotein/nucleic acid complexes prior to acid phenol-chloroform separation. Measles nucleoprotein forms very stable associations with measles RNA (Andzhaparidze *et al.* 1987) and it is possible that the high salt concentrations used for RNA isolation do not fully dissociate measles nucleoprotein from measles RNA. As a consequence, measles RNA may remain associated with measles nucleoprotein in the organic phase after acid phenol-chloroform separation and not detected in total RNA purified from the aqueous phase. Alternatively, measles nucleoprotein may remain associated to measles RNA in the aqueous phase following acid-phenol separation. Bound measles nucleoprotein may then inhibit reverse transcription of measles RNA, reducing the sensitivity of the measles RNA detection method.

Measles RNA mutations. As discussed in Chapter 3, mutations accumulate in genomic measles RNA during persistent measles virus infection (Cattaneo *et al.* 1989). Mutations in measles matrix (Hirano *et al.* 1992; Hirano *et al.* 1993) and fusion proteins (Schmid *et al.* 1992) are thought to be partly responsible for the establishment of viral persistence. As viral envelope proteins are not required for persistent measles infection, mutations also accumulate in the genes encoding these proteins.

RNA amplification techniques require efficient hybridisation of oligonucleotide primers to target RNA sequences. In the case of highly mutated RNA targets, any mismatches occurring at the 3' end of the oligonucleotide primer may lead to inefficient primer extension, preventing optimal amplification of measles RNA sequences.

As a consequence of the high mutation rate within the matrix, fusion and haemagglutinin genes of persistent measles virus, transcription of these genes is often restricted, leading to a characteristically steep gradient of measles mRNA expression (Sidhu *et al.* 1994). Therefore, the abundance of mRNA encoded by genes towards the 5' end of the measles genome (measles P, M, F, H, and L-genes) is significantly reduced, possibly below the sensitivity limit of a detection methodology. However, genomic (rather than messenger) measles H-gene RNA

was used as a target for amplification in experiments described in Chapter 8 and so the presence of a measles mRNA transcription gradient should not have interfered with measles genomic H-gene analysis.

On the other hand, expression of measles N-gene mRNA is often normal in persistent infections (Sidhu *et al.* 1994). Moreover, measles nucleoprotein, phosphoprotein and large proteins are essential for measles RNA replication and packaging, therefore mutations rarely accumulate in these genes. Although mutations may affect the ability to detect measles M, F and H-gene RNA in persistently infected tissue, measles N-gene RNA should be detectable if enough infected cells are present in the samples analysed.

Based on the results in Chapters 6 and 9, and those of other studies, various measles RNA species (including N and H-gene RNA) are detectable in persistently infected SSPE tissue using NASBA, RT-PCR (Godcc *et al.* 1990; Katayama *et al.* 1995) and RT-PCR followed by *in situ* hybridisation (Esolen *et al.* 1995; Isaacson *et al.* 1996). It is therefore very unlikely that measles RNA is not detectable in clinical samples of patients with IBD purely because the measles RNA is highly mutated.

11.1.4. Conclusion

The results of this project (supported by results of other groups) offer no supportive evidence for the involvement of measles virus in the aetiology of IBD. However, it remains possible that a persistent measles virus infection is present in IBD tissue and that the measles RNA detection methods employed in this and other studies were of insufficient sensitivity to permit measles RNA detection. Further work may lead to the detection of measles RNA in IBD tissue via more sensitive methodologies.

The presence of measles antigens in intestinal tissue of patients with Crohn's disease requires confirmation with an affinity-purified measles polyclonal antibody in order to reproduce the data of previous studies. A panel of measles monoclonal antibodies, directed against different measles antigens (or different epitopes of the same antigen), should also be used to try to obtain similar staining patterns which would provide convincing evidence for the presence of measles antigens in IBD tissue. Using a range of monoclonal antibodies would

reduce the possibility of obtaining false-positive results due to antibody cross-reactivity as suggested by the study by Iisuka and Masamune (1997).

The specificity of polyclonal measles antibody sera used for immunohistochemical studies (Wakefield *et al.* 1989; Lewin *et al.* 1995; Ekbom *et al.* 1996; Daszak *et al.* 1997) should be determined by Western blot analysis with the human antigen expressed in the phage λ library described by Iisuka and Masamune (1997) and discussed above. This would determine whether these polyclonal cross-react with the same human antigen which MAS 182r appears to. In addition, double immunohistochemical staining of IBD tissue sections, using monoclonal antibody 4F12 (directed against the human protein discussed above), and each measles polyclonal antibody sera, would provide information concerning the specificity of the measles antigen staining in IBD tissue.

The detection of measles antigen in IBD tissue may reflect long term retention of antigen-antibody complexes as a function of normal immunological memory, rather than showing a causal relationship between measles antigen and chronic inflammation. Therefore future immunohistochemical studies for measles antigens in IBD tissue should include large numbers of age-matched controls to determine the significance of any positive staining in IBD samples.

Epidemiological data suggests that atypical exposure to measles virus is a risk factor for the later development of IBD. Persistence of measles virus may not be a prerequisite for the development of chronic intestinal inflammation. As discussed above, it is possible that molecular mimicry occurs between measles virus and a human protein leading to autoimmune-mediated chronic inflammation later in life. Therefore the possible absence of measles virus in IBD tissue does not reduce the significance of the measles virus and IBD epidemiological findings.

11.2. MEASLES, MUMPS AND RUBELLA RNA AND AUTISTIC ENTEROPATHY

No measles, mumps or rubella RNA was detected in any of the clinical samples analysed in our laboratory from paediatric patients with autistic enteropathy.

Immunohistochemical staining, using measles nucleoprotein polyclonal antibody sera (RAd 68), has indicated the presence of measles nucleoprotein in biopsy sections from these patients (Wakefield *et al.* 1998a). As is the case for the detection of measles virus in IBD, there are significant discrepancies between the positive immunohistochemical staining for measles antigen and the negative RT-PCR results for measles RNA. Three possibilities may explain this discrepancy: measles virus is not present; measles antigens are present in the absence of measles RNA; or measles RNA is present but not detectable using the methods described in this project.

11.2.1. Measles virus is not present

RAd 68 was derived from mice immunised with purified recombinant measles nucleoprotein expressed using an adenovirus vector. The purification of recombinant measles nucleoprotein prior to immunisation is important: a significant proportion of infantile gastroenteritis in the UK is caused by adenoviruses (Bryden, 1997), it was essential that no adenovirus antibodies were present in RAd 68 sera.

However, the polyclonal antibody sera may have cross-reacted with a human protein, as described by Iisuka and Masamune (1997) for the monoclonal antibody MAS 182r discussed above.

On the other hand, an adenovirus monoclonal antibody did not give any positive staining in tissues positive for RAd 68, indicating that this antibody did not recognise adenovirus antigens in the tissue samples analysed (Wakefield *et al.* 1998a). The antibody sera specifically bound to recombinant measles nucleoprotein on Western blot analysis and stained SSPE and measles-virus infected Vero cells by immunohistochemistry, confirming the presence of specific measles nucleoprotein antibodies in this polyclonal sera. In addition, the antibody sera used specifically stained follicular dendritic cells in lymphoid

tissue, a common site of measles virus infection.

These findings suggest that the immunohistochemical detection of measles nucleoprotein in biopsies from affected patients represents a real result and is not a result of non-specific antibody binding.

11.2.2. Measles antigens are present in the absence of measles RNA

Measles antigen retention. It has recently been postulated that a mechanism for the longevity of the immune response to measles virus could involve persistence of measles virus antigens but not measles RNA (Griffin *et al.* 1994). Follicular dendritic cells in lymphoid tissue may retain measles antigens for long periods following a measles virus infection and possibly measles vaccination.

In 1979, Tew and Mandel demonstrated prolonged retention of ^{125}I -labelled human serum albumin in lymphoid follicles of mice footpads after antigen had been degraded and cleared from other sites. Calculating the half-life of these antigen, it was shown that it may be able to persist for many years, possibly accounting for B-cell memory. Moreover, only a minority of dendritic cells retained antigen as shown by discrete staining of isolated cells in lymphoid tissue. the

Szakai *et al.* (1988) identified a mechanism of antigen retention in follicular dendritic cells involving antibody-antigen complexes. Such complexes are found in vesicles called iccosomes and these may be responsible for both antigen presentation to T and B cells in lymphoid tissue and antigen retention in the form of convoluted dendrites. At sites of measles virus infection, dendritic cells may endocytose viral antigens (or antibody-antigen complexes) or become infected. Migration of such cells to lymphoid tissue leads to exposure of viral antigens, either as immune complexes within iccosomes released from dendrites, or via MHC class I-restricted antigen presentation. Iccosomes released from dendritic cells are endocytosed by macrophage and B-cells leading to MHC class II-restricted expression of viral antigens and stimulating CD8⁺ cytotoxic lymphocytes. MHC class I-restricted expression of viral antigens on dendritic cells stimulates CD4⁺ T cells which recruit B cell help. In addition, some dendritic cells retain iccosomes within highly convoluted dendrites, leading to the persistence of viral antigens for many years. In this way, memory B cells

may be stimulated, conferring long-term immunity. Such a phenomenon has been observed with human immunodeficiency virus (Tacchetti *et al.* 1997), with viral antigens being trapped in follicular dendritic cells of lymphoid tissue.

Tew *et al.* (1990) have postulated that, following exposure to an antigen, convoluted follicular dendritic cells retain antigen-antibody complexes for long periods of time. When antibody levels decline, a feedback mechanism exposes antigen, stimulating B cells to produce more antibody. In this manner, retained antigen levels are slowly exhausted over time to maintain antibody and memory B cell levels. The ratio of retained antigen:antibody in these complexes determines the immunogenicity of these complexes and it is possible that abnormally high levels of retained antigen lead to immunopathogenic events, stimulating chronic inflammation.

This mechanism of antigen persistence may explain the phenomenon of lifelong immunity to measles virus and may also explain the detection of measles nucleoprotein in isolated dendritic cells of affected children, in the apparent absence of measles RNA. However, there is much debate as to whether antigen persistence is a requirement for long term immunological memory (Doherty *et al.* 1996) and the mechanisms of lifelong immunity to measles virus are currently under investigation in other laboratories.

If retention of measles antigens as part of immune complexes in dendritic cells is a normal function of long term immunity to measles virus, then the detection of measles antigens in these cells may not be surprising and may even be unrelated to the chronic colitis observed in affected children. With this in mind, the best way to determine the significance of any positive measles antigen immunohistochemical staining in affected children would be to examine, in parallel, tissue from large numbers/normal control children. It is essential that control children are age-matched with affected children as the amount of measles antigen retained in lymphoid tissue may decrease over time as suggested by the study of Tew and Mandel (1979).

As discussed above, it is possible that measles DNA may be present in affected patients, leading to the expression of measles antigens. The transient expression of measles RNA may account for the difficulties encountered in its detection.

11.2.3. Measles RNA is present but not detectable

As described above for IBD tissue, it is possible that measles RNA is present in clinical samples from patients with autistic enteropathy, but below the sensitivity of the detection methods employed. The sensitivities of the measles N- and H-gene RT-PCR detection methods used in this study were not accurately quantified although measles N- and H-gene RNA was detected in positive control SSPE tissue using these methods. It remains possible therefore, that a persistent, low copy-number measles virus infection was present but not detectable in the samples analysed.

11.2.4. Conclusion

It is possible that a persistent measles virus infection is present in intestinal tissue of patients with autistic enteropathy, but below the sensitivity of the detection methods employed. However, the combined data from immunohistochemistry and RT-PCR studies raise the interesting possibility that measles antigens may be retained in isolated follicular dendritic cells within lymphoid tissue in the absence of measles RNA many years after exposure to wild-type or vaccine strain measles virus.

Griffin and her co-workers are currently studying the mechanisms of the longevity of immunity to measles virus and such studies may help to determine whether i) the presence of measles antigens in lymphoid tissue is an integral part of this mechanism, or ii) measles antigens are involved with immunopathology, leading to chronic inflammation and regressive autism observed in these patients.

Iisuka and Masamune (1997) and Lui *et al.* (1995) have raised doubts concerning the specificity of antibodies used to detect measles antigens in IBD tissue. Although a different polyclonal antibody was used in this study, it may be necessary to repeat the measles immunohistochemistry experiments on tissue from affected children using an affinity-purified measles polyclonal antibody. In addition, a panel of measles monoclonal antibodies, directed against different measles antigens (or different epitopes of the same antigen), should also be used to try to obtain similar staining patterns which would provide convincing evidence for the presence of measles antigens in affected children. Such work is

currently underway in our laboratory.

11.3. FUTURE WORK

11.3.1. Measles virus and IBD

Given the results of Iisuka and Masamune (1997), concerning measles antibody cross-reactivity, it is necessary to re-evaluate the original immunohistochemical findings concerning the presence of measles RNA in IBD tissue. If the original data are confirmed, these immunohistochemical studies have detected measles antigens in the intestine of non-IBD patients (Lewin *et al.* 1995; Daszak *et al.* 1997), raising the possibility that the measles antigens detected are involved with long term immunological memory and unrelated to chronic intestinal inflammation. Therefore, studying many more non-IBD intestinal tissue may reveal the significance of detecting measles antigens in IBD tissue.

If measles RNA is present in IBD tissue, then it is present below the sensitivity limits of the methods described in this and previous studies. Three methodologies may increase the detection sensitivity of measles RNA in IBD tissue: microdissection of Crohn's disease granulomas; isolation of human intestinal microvascular endothelial cells (HIMECs); and *in situ* RT-PCR.

Microdissection of granulomas. Haematoxylin and eosin staining can be used to locate the position of granulomas in frozen or paraffin-embedded Crohn's disease tissue. These granulomas may then be isolated from measles antigen positive sections of tissue using a dissecting microscope and either used directly in a measles RT-PCR reaction, or used for total RNA isolation prior to measles RNA amplification. Such a method may significantly increase the ratio of measles:non-measles RNA present in the reaction mixture and may lead to the detection of measles RNA previously undetectable in whole tissue.

Microdissection techniques have been described for the isolation of tumour cells prior to PCR studies to determine the presence of particular gene mutations (Hedrum *et al.* 1912; Wagner *et al.* 1995). In addition a microdissection technique, coupled with RT-PCR has been used for the detection of hepatitis C virus RNA in different liver cell types (Saito *et al.* 1997). Such a method may be adapted for the isolation of Crohn's disease granulomas prior to RT-PCR studies for measles RNA to improve the measles RNA detection sensitivity.

Isolation of HIMECs. Recently, HIMECs have been isolated and cultured from resection tissue of patients with IBD (Haraldsen *et al.* 1995). As measles antigens have been detected in these cells in IBD tissue, they may represent an enriched source of any measles RNA present and this enrichment may permit the detection of measles RNA previously not detectable in IBD tissue samples. However, persistently infected HIMECs may be less likely to survive isolation from diseased tissue and may therefore be under represented in cell culture. Experiments to isolate HIMECs from IBD tissue as a source of RNA for measles RT-PCR are currently underway.

In situ PCR. If only several endothelial cells are infected with measles virus within a resection tissue sample, then total RNA from these samples may contain measles RNA below the sensitivity limits of conventional measles RNA amplification methods. *In situ* RT-PCR, followed by *in situ* hybridisation using a probe specific to the PCR product, may be used to identify very few infected cells within a tissue section and can also determine the infected cell type. An advantage of this technique is that it can be applied to formalin-fixed and paraffin embedded archival tissue.

Both *in situ* PCR and *in situ* RT-PCR have been used to detect a number of DNA (Berger *et al.* 1997; Foreman *et al.* 1997) and RNA (Schmidtmayerova *et al.* 1996; Ramakrishnan *et al.* 1996) templates, respectively. Measles RNA has also been detected using *in situ* RT-PCR (Esolen *et al.* 1995; Ray *et al.* 1996; Isaacson *et al.* 1996) and two of these studies (Esolen *et al.* 1995; Isaacson *et al.* 1996) have reported the identification of measles RNA in endothelial cells, previously undetectable by *in situ* hybridisation. Therefore this technique may help improve the detection of any measles RNA in sections of IBD tissue.

Targeted in situ PCR. Pan *et al.* (1997) have recently developed a method of *in situ* PCR whereby tissue sections are cut onto glass coverslips, the tissue of interest is isolated using a diamond marker and the resulting sliver of glass (containing the tissue of interest) is used in a conventional RT-PCR reaction under mineral oil in an Eppendorf tube. Amplification products can be analysed by gel electrophoresis of the PCR reaction supernatant and the *in situ* location of

PCR products can be determined by *in situ* hybridisation on the tissue on the glass sliver. Preliminary work using a modified method for RNA detection has shown that this method is capable of detecting measles RNA in SSPE tissue sections. Work is currently underway using this method to detect any measles RNA present in Crohn's disease granulomas. In addition, this method should be capable of detecting any measles DNA present in Crohn's disease tissue as discussed above.

11.3.2. Measles virus and autistic enteropathy.

Measles RNA. As discussed above, the failure to detect measles RNA in intestinal tissue from these patients may be a result of inadequate measles RNA detection sensitivity. Even though the natural RNA transcription gradient of the measles genome (Sidhu *et al.* 1994) would favour the detection of measles N-gene RNA over other measles RNA species, measles H-gene RNA may be a more appropriate target for measles RNA detection. For this reason, experiments are currently underway in our laboratory to optimise measles H-gene RT-PCR. Additionally, analysis of RNA from clinical samples of patients with autistic enteropathy for measles H-gene RNA work is currently underway in an independent laboratory (Dr H. Kawashima, Tokyo Medical School, Japan; personal communication).

Targetted *in situ* RT-PCR for measles RNA in intestinal tissue (described above) may also permit the detection of previously undetectable measles RNA.

Measles antigens. It remains a possibility that measles RNA is not present in the intestinal tissues analysed from these patients and therefore the detection of measles antigens in these tissues (Wakefield *et al.* 1998a) needs to be confirmed. Immunohistochemistry using a range of monoclonal, or affinity purified polyclonal antibodies, specific to measles antigens, may be used to confirm the presence and location of measles antigens within lymphoid tissue. In addition, analysis of non-inflamed tissue from the same patients and tissue from non-inflammatory age-matched control patients may reveal the significance of any positive findings.

Moreover, immunohistochemistry for other viral antigens (such as mumps or

rubella) may reveal if antigen retention by isolated follicular dendritic cells is a normal part of long term immunity to viruses in general.

Measles DNA. As discussed previously, a study by Zhdanov (1975), supported by the results of a recent study (Klenerman *et al.* 1997), has raised the possibility that measles may exist in a persistent DNA form. Such latent measles DNA (possibly integrated into the host genome) may generate immunopathogenic amounts of measles antigen, leading to chronic colitis. Experiments are underway in our laboratory to determine whether measles DNA is generated in cell cultures persistently or acutely infected with either measles virus or measles, mumps and rubella viruses derived from MMR vaccine preparation. Depending on the results of these experiments, DNA may be isolated from clinical samples of affected children (and from IBD samples), purified and RNase-treated, and used as a template for measles PCR to determine the presence of measles DNA.

Such experiments are interesting both in the context of mechanisms of measles virus replication and persistence, and in the context of the mechanism of long term immunological memory to measles virus.

Although the results of this project offer no supportive evidence of an association between measles virus and chronic gastrointestinal inflammation, the future work described here may provide further insights into the relationship.

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CORRECTIONS

PAGE LINE

11.	30	“has been recently” instead of “have been recently”
21.	Fig.1.1.	“Livingstone” instead of “Livinstone”
27.	6	“Gutensohn and Cole” instead of “Gutersohn and Cole”
29.	8	“Disease pathogenesis.” instead of “diseased pathogenesis”
42	18	“Mycobacterial” instead of “mycrobacterial”
44	3,14	“ <i>Lysteria monocytogenes</i> ” instead of “ <i>Lysteria monocytogenese</i> ”
44	9	“presence of <i>Listeria</i> ” instead of “presence <i>Listeria</i> ”
44	21	“represents opportunistic...” instead of “represent opportunistic”
46	5	“immunodeficient” instead of “immunodefficient”
46	8	“and to the presence of pro-inflammatory cytokines” instead of “and pro-inflammatory cytokines”
47	5	“a range of tissues” instead of “a range for tissues”
47	16	“Koplik spots” instead of “Koplick spots”
49	4	“Upsala” instead of “Upsalla”
49	25	“has shown concurrent exposure to measles and mumps” instead of “has shown concurrent measles and mumps infections”
51	9	“increased dramatically” instead of “increased was dramatically”
51	10	“decided to study” instead of “decided the study”
57	Fig.2.1.	Scale of bars:
62	26	“involved with the” instead of “involve with the”
66	20	“aphthoid ulcers” instead of “apthoid ulcers”
70	2,3	Swap Bansil and Dhib-Jalbut references with Schulz reference
71	13	“genomic RNA instead of “geneomic RNA”
71	18	“vesicular stomatis virus” instead of “vesicular somatis virus”
74	11	“syncytia formation” instead of syncytia formation”
75	15	“characterised by” instead of “characterised my”
81	12	“immunohistochemical” instead of “immmunohistochemical”
88	12	“and has a similar” instead of “and a similar”
90	6	“RNA in PBMCs” instead of “RNA PBMCs”
98	12	“electrophoresis” instead of “lectrophoresis”
99	10	“phytohamagglutinin” instead of “phytohaemagluttinin”
99	16,17,18, 26,32,33	“medium” instead of “media”
100	2,5,8,9,13 24,26,31	“medium” instead of “media”
100	10	“cocultures mixed” instead of “cocultures. mixed”
100	17	“Cells derived from” instead of “Cell derived from”
100	25	“medium was replaced” instead of “medium media was replaced”
102	24	“deproteinised” instead of “deprotienised”
103	1	“resuspended in” instead of “resuspend in”
107	16	“gel electrophoresis” instead of “gel electrophoresism”

107	24	"electrophoresis was carried out at" instead of "electrophoresis at"
108	10	"Membranes were stored" instead of "Membranes stored"
111	1,16	"deproteinised" instead of "deprotienised"
111	12	"were phosphorylated" instead of "was phosphorylated"
112	7,12	"deproteinised" instead of "deprotienised"
119	2	"generated were used" instead of "generated was used"
125	24	"detect as few as" instead of "detect as few as"
132	14	"was analysed" instead of "was alalyzed"
133	1	"study were to" instead of "study was to"
136	Fig.6.2.	"section 4.2.9." instead of "section 4.2.8."
141	2	"characterize" instead of "charcterize"
149	26	"products are used" instead of "products are are used"
150	5	Delete sentence "In experiments to determine..."
150	9	"guanidine" instead of "gaunidine"
152	Title	"competitive" instead of "competetive"
152	5	"Oligonucleotide" instead of "oligonucleotide"
153	3/4	"transcripts into" instead of "transcripts was spiked into"
168	31	"messenger" instead of "messenger"
169	6	"...mumps or rubella could be detected." instead of "mumps or rubella."
173	8	"characterize" instead of "charcterize"
174	7	"methods' general" instead of "methods general"
174	11	"intestinal or SSPE tissue" instead of "intestinal of SSPE tissue"
177	Fig.9.2.	"Lane 11 contains" instead of "Lane 11 conains"
177	Fig.9.2.	"control reaction" instead of "control reactions"
178	14	"to achieve" instead of "to achiev"
180	Fig.9.3.	"respectively" instead of "receptively"
184	17	"could have occurred" instead of "could have occurs"
194	3	"No samples" instead of "None samples"
203	17	"characterize" instead of "charcterize"
204	1,3,9,10,13	"serum" instead of "sera"
205	15	"serum" instead of "sera"
205	21	"results of these studies suggest" instead of "results suggest of these studies"
206	14	"This explanation" instead of "this explanation"
208	10	"detected in MMR" instead of "detected MMR"
210	28	"present in Crohn's" instead of "present Crohn's"
211	2	"dissociation of nucleoprotein" instead of dissociation nucleoprotein"
211	32	"messenger" instead of "messenger"
215	12	"of this antigen" instead of "of these antigen"
216	26	"numbers of normal" instead of "numbers normal"
222	20	"described" instead of "desribed"

